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Defining the role and control of the *lpxO* gene in *Klebsiella pneumoniae*

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Certification and acknowledgements

This is to certify that the work presented here for the degree of Master of Science by Research in Infectious Diseases is the result of my own investigation under the internal supervision of Dr Thamarai Schneiders, whom I wish to thank for her invaluable guidance, drive and encouragement during the course of this project. Furthermore, I am grateful for the efforts of my personal tutor, Dr Kim Picozzi; for her support and advice throughout the year. I also acknowledge the help of fellow members of the Schneiders group with whom I had the pleasure of working, and thank Alexia Kalligeraki for her enduring patience. Lastly and most importantly, I dedicate this thesis to my parents Christine Usher and Kevin Usher, without whom this opportunity would not have been possible.

Name…….Ben Usher

Signature……

Abstract

Antimicrobial resistance (AMR) is fast becoming one of the greatest challenges to human mortality this century. In recent years the number of reported infections by multi-drug resistant (MDR) bacteria has seen a rapid rise. As a result, the efficacy of currently used antibiotics is decreasing, with infections by MDR bacteria increasingly difficult to treat. In more and more cases, the use of last-line drugs is becoming necessary. However, this in turn has resulted in increasing reports of last-line drug resistance, presenting a dangerous cycle that leaves us potentially short of treatment options. As a result, elucidating the intrinsic resistance mechanisms that pathogens employ, and their importance in generating an AMR phenotype, is essential to developing a comprehensive and effective antimicrobial strategy. Furthermore, identifying specific resistance mechanisms and their contribution to AMR presents potential targets for inhibitory drugs in combination with existing antimicrobials.

This project looks at the role of the transcription regulator RamA in generating an AMR phenotype in the Gram-negative bacterium *Klebsiella pneumoniae*. *K. pneumoniae* is a major cause of nosocomial infections in the immunocompromised, with MDR isolates resistant to the last-line drug colistin rapidly increasing in prevalence. *K. pneumoniae* RamA regulates genes associated with virulence and resistance, able to modulate outer membrane permeability through altered influx and efflux. Overexpression of *ramA* has also been shown to upregulate the dioxygenase LpxO, a lipid A modifying enzyme associated with increased resistance to colistin, polymyxin B, and the human cationic antimicrobial peptide LL-37. Our study seeks to define the relative importance of RamA and LpxO in relation to polymyxin B, colistin and LL-37; specifically how RamA-mediated *lpxO*-overexpression confers this phenotype. In this regard, we have performed MIC and relative survival assay studies with wild-type *K. pneumoniae*, *ramA*- and *lpxO*-overexpressing strains, in order to define the role of these genes in the relevant phenotypes.

Lay Summary

Antimicrobial resistance (AMR) is an immediate danger to public health worldwide, threatening the delivery of effective treatment against a range of infectious diseases. Due to the rapid development of resistance among pathogens, we are stumbling into a situation where demand for effective treatment outweighs supply; where without a change of strategy, currently routine surgeries will soon present high risk of potentially lethal infection.

The bacterium *Klebsiella pneumoniae* is a major cause of respiratory and blood stream infections in long-term healthcare settings. It is an increasingly important pathogen worldwide due to the emergence of multi-drug resistant strains capable of surviving in the face of antimicrobial treatment. Significantly, strains have recently been identified that are resistant to colistin, a “last-resort” drug used to treat already resistant infections.

Our project focuses on intrinsic resistance in *K. pneumoniae*; the ability of the bacterium to develop resistance under antimicrobial pressure due to inherent structural and functional properties. RamA is an intrinsic *K. pneumoniae* protein, previously demonstrated to drive alterations to the bacterial surface and affect bacterial permeability. This prevents antimicrobial recognition, binding, and cell entry by host immune factors and antibiotic drugs. LpxO is an enzyme capable of changing outer membrane structure, linked with increased survival after antimicrobial challenge. Whilst its activity has been shown to be regulated by RamA, other factors also influence its control.

The roles of RamA and LpxO in AMR are important for bacterial infection and resistance to antibiotics. Our work looks to define the relative importance of RamA and LpxO in contributing to *K. pneumoniae* AMR; to better understand how RamA controls the *lpxO* gene to modify the cell surface of *K. pneumoniae*.

Contents

Key of abbreviations

- **Δ =** deletion (for example: "Δ*lpxO*" corresponds to a "*lpxO* deletion"
- **<>Km** = gene replaced by a Km cassette (for example: "<lpxO>Km" means that the *lpxO* gene has been replaced by a Km cassette)
- **3GC** = third-generation cephalosporins
- **Amp** = ampicillin
- **AMR** = antimicrobial resistance
- **bp** = base pairs
- **CAMP** = (cationic antimicrobial peptide)
- **Cm** = chloramphenicol
- **FRT** = *Flp* recognition target
- **IM** = inner membrane
- **Km** = kanamycin
- **LPS** = lipopolysaccharide
- **MBL** = metallo-beta-lactamase
- **MDR** = multi-drug resistance/resistant
- **MIC** = minimum inhibitory concentration
- **MW** = molecular weight
- **OM** = outer membrane
- **PAMP** = pathogen-associated molecular pattern
- **PRR** = pattern recognition receptor
- **PxB** = polymyxin B
- **^{R}** = resistance/resistant (for example: "Km^{R}" refers to kanamycin resistance or a kanamycin-resistant gene)
- **RSA** = relative survival assay
- **^{S}** = sensitivity/sensitive (for example: "Km^{S}" means either kanamycin sensitivity or a kanamycin-sensitive gene
- **sdH_2O** = sterile distilled water
- **SN** = supernatant
- **spp.** = species
- **Tc** = tetracycline
- **TCS** = two-component system
- **w/t** = wild-type

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1. Introduction

1.1. Antimicrobial Resistance: a Global Crisis

Antimicrobial resistance (AMR) presents an immediate danger to global public health, threatening the delivery of effective prevention and treatment to a broad range of infectious diseases. Antimicrobial drug development has failed to keep pace with a constantly evolving assortment of resistant pathogens, leading to limited options for patient treatment [6]. In the absence of effective antimicrobials, the ability to conduct currently routine surgeries will be severely compromised, whilst young, healthy individuals will find themselves vulnerable to what were previously easily treatable infections. On our current heading, and without a concerted change of strategy, we are facing a post-antimicrobial period in which common infections will pose a high risk of death.

1.1.1. An overview of AMR

Beginning with the original discovery and development of antimicrobials, the fight against pathogens and infectious diseases took a major swing in favour of public health. Many infections with previously deadly pathogens quickly became easily treatable [7, 8]. Penicillin for example, discovered in 1928 by Alexander Fleming [9], became a commonly used treatment against *Staphylococcus aureus* infection after World War II [8]. Nonetheless, as soon as the early 1950s, penicillin-resistant *S. aureus* strains had emerged that were being regularly isolated in clinical settings [8].

Resistance of pathogens to antimicrobials is a natural process occurring over time, part of the evolutionary development by which these organisms persist and survive in the face of selective pressure from unfavourable environments. However, constant exposure to antimicrobials leads to continual selective pressure, accelerating the evolution of resistance by forcing pathogens to adapt and survive [10]. In recent decades, due to widespread use and misuse of antimicrobial drugs in human healthcare [11], as well as animal management and food production [12, 13], the threat of AMR has emerged as a major and immediate public health crisis.

One of the key factors contributing to AMR is antimicrobial use in animal husbandry. Globally, it has been estimated that two thirds of all antibacterial drugs produced each year are used in animal care [14]. They are often applied as metaphylactics *en masse* to livestock at the first sign of illness [15], and this liberal distribution is likely an important contributor to the selection and spread of environmental AMR [16].

Especially worrying is that the classes of antibacterial drugs administered in animal care include all those important for human medicine [17], whilst some agents used are considered by the World Health Organisation (WHO) to be of critical importance to humans [18].

One of the major challenges accompanying increasing rates of AMR is changing global demographics: populations are ageing, leading to a greater number of people with weakened immune systems, the body's first and most effective defence against infection [19]. The elderly also tend to suffer more from co-morbidities, resulting in an increased vulnerability to infection; even from non-resistant pathogens and when effective antimicrobials are still available. As resistant pathogens become a regular occurrence and existing drugs become less successful at clearing infection, these populations will face an even bigger risk of serious illness.

Furthermore, with a greater prevalence of AMR comes a significant threat to the ability to deliver commonplace health services. Antimicrobials are essential prophylactics and treatments used to quickly and effectively protect from and fight infections; preventing the onset of potentially fatal diseases in vulnerable patients and allowing operations to be performed with low risk of infection [20]. They are essential for routine surgeries, such as caesarean sections or hip replacements, to protect patients against infection. They are also a fundamental component of chemotherapy for cancer treatment and organ transplants, due to the treatment's need to repress the body's immune system and therefore remove its inherent, natural defence. As these antimicrobials become ever more ineffective at battling resistant infections, the risks associated with previously everyday treatments and surgeries will become too high.

Worryingly, we are reaching a point where even resistance to last-line drugs is beginning to emerge [20]. These are antimicrobial drugs used as a last resort provided all other options have been exhausted, which are both expensive and often accompanied by adverse side effects. A report from January 2017 stresses the significance of the escalating nature of AMR: an elderly patient in the United States who died in September 2016 of septic shock was infected with a strain of bacteria resistant to all antimicrobial drugs available, including last-line drugs [21].

The ability of pathogens to acquire cross-resistance to host immune defences and antimicrobial drugs is also an alarming prospect [22-24]. Healthy, young populations

with fully functioning immune systems may have to face the possibility of potentially debilitating disease as a result of previously treatable infections. When all of these factors are taken into consideration, it is easy to see how the estimated numbers of global deaths caused by AMR annually rises from seven hundred thousand at present to approximately ten million by 2050 [3] (Figure 1). As a result, a collaborative international effort is essential in order to fight back against the looming AMR crisis.

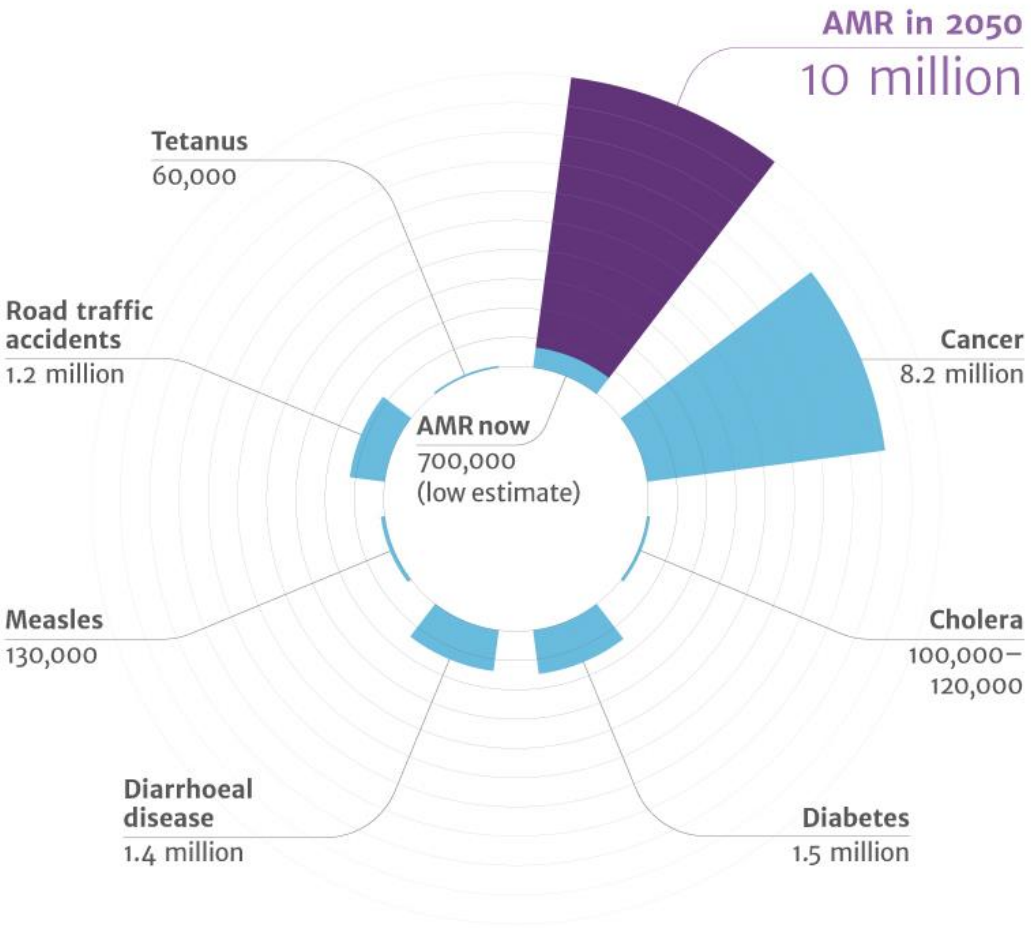

Figure 1. Numbers of attributable deaths per year compared between different causes. Currently, AMR is estimated to result in 700,000 deaths per year; a significant figure but one which pales in comparison to fatalities from cancer. If strategies to combat AMR remain unchanged, it is estimated to lead to approximately 10 million deaths per year by 2050. *Adapted from the Review on Antimicrobial Resistance. Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations. 2014* [3].

1.1.2. Economic fallout from AMR

The rapid emergence of AMR has had impacts beyond solely patient welfare; the economic effects are also considerable (Figure 2). Patients suffering resistant infections are hospitalised for longer, draining medical resources and potentially requiring more costly medical interventions. A major reason for rising costs is treatment failure, often caused initially by sub-optimal dosing or incorrect diagnoses. Increasingly, doctors are turning to last-line drugs to treat resistant infections. These are more expensive, have greater adverse effects, and may be inaccessible in lower-income regions [25].

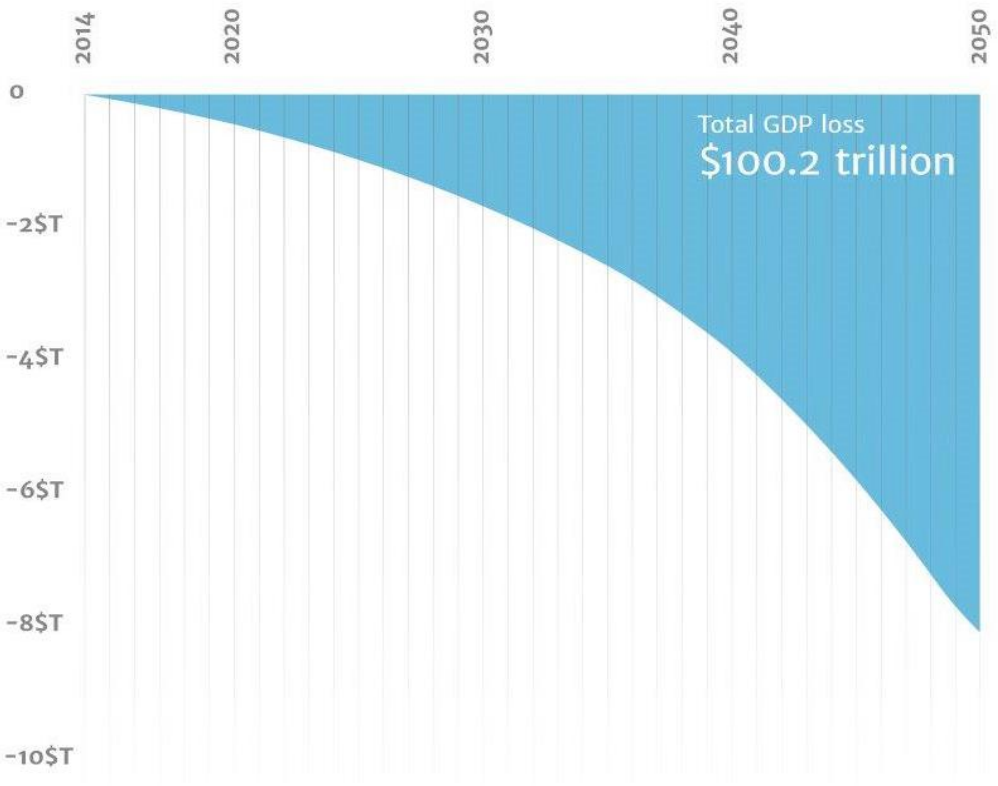

Figure 2. Predicted cumulative world GDP loss in US$ trillions as a result of AMR. The steadily accumulating global GDP loss, represented by the shaded blue area, conservatively estimates that by 2050 the global economy stands to lose approximately US$100.2 trillion as a result of AMR. Higher risk of infection may lead to rejection of surgical operations; reductions in tourism and trade might result from global transmission fears; decreased efficacy of current drugs could lead to reduced purchase and use. *Adapted from the Review on Antimicrobial Resistance. Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations. 2014* [3].

Economic productivity is also indirectly affected by sick leave; the lengthier hospitalisation of workers due to resistant infections causes a knock-on effect on economic output. Although this particular argument should be considered in context – the majority of resistant infections occur in the elderly and those with co-morbidities, patients who are unlikely to be contributing significantly to the workforce [25] – the combination of these factors considerably influences the direct and indirect impact of AMR on more than just public health.

1.1.3. Investment in AMR research is essential

There is an urgent need for greater investment into novel research to better understand the development of resistance in pathogens, optimise existing antimicrobials, and develop new medicines and preventative measures with which to strengthen global health systems. However, in recent decades there have been no significant new drug discoveries (Figure 3), and currently there are very few novel drugs in the development pipeline, with economic incentives for large pharmaceutical companies lacking [26]. Indeed, a look at the March 2016 product pipeline for GlaxoSmithKline indicates that of the approximately one hundred pharmaceuticals and vaccines in development, fourteen are related to HIV and infectious diseases, and only one is for the treatment of bacterial infections [27]. Furthermore, Pfizer's pipeline lists a total of 94 products as of November 2016, but no antimicrobial drugs in development [28].

Bridging the gap between preclinical and clinical development is an area of concern for many drug companies; costly and highly-regulated clinical trials may fail or simply not meet the regulatory requirements imposed, whilst the short-term dosing of most antimicrobials, and the requirement for inexpensiveness and limited application, means returns on investment are lower than most chronic disease medications [29, 30]. Consequently, investment in antimicrobial development has faltered, thereby contributing to the current AMR crisis and leaving treatment options limited.

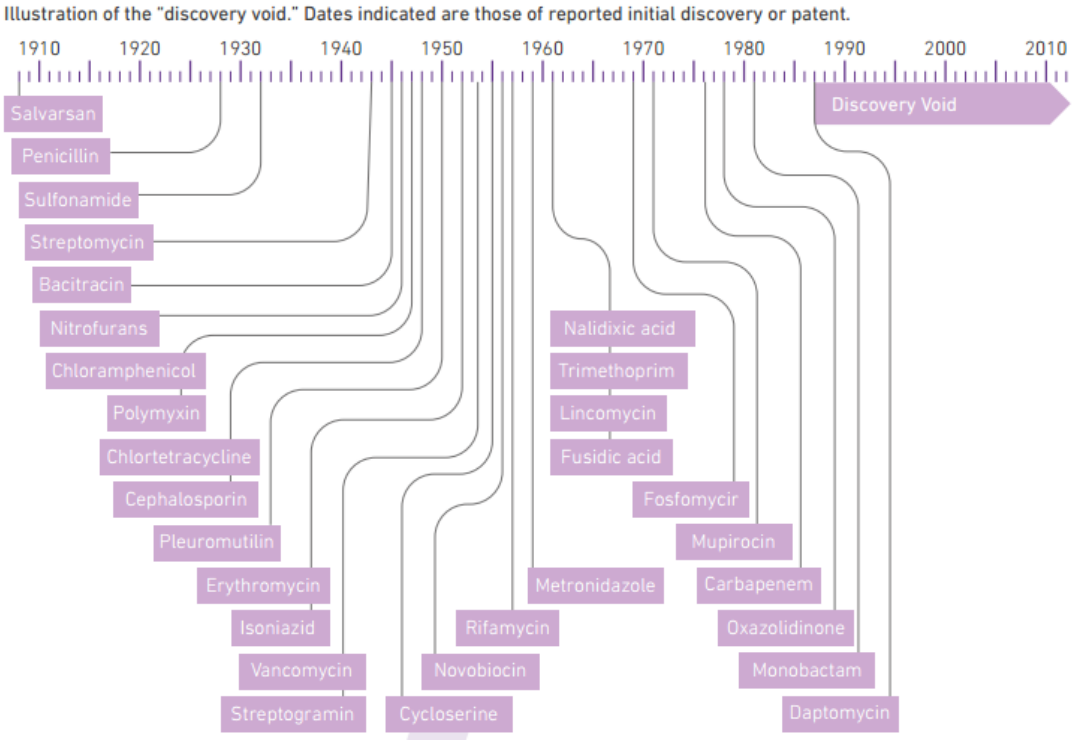

Figure 3. Discovery timeline of distinct antibacterial drug classes. In recent decades there has been a dearth of research and investment by major pharmaceutical companies into novel antibacterial drugs, with no new drug classes identified and developed since 1987. *Adapted from the WHO Antimicrobial Resistance Global Report on Surveillance, 2014* [2].

1.2. *Klebsiella pneumoniae*

Klebsiella pneumoniae (*K. pneumoniae*) is a Gram-negative rod-shaped bacterium belonging to the family *Enterobacteriaceae*, which also includes pathogens such as *Escherichia coli* (*E. coli)* and *Salmonella* species (spp.). Members of the *Enterobacteriaceae* family typically reside as part of the gut microbiota of humans and animals [31-33], whilst some can reside naturally in the environment [34-37]. Some *Enterobacteriaceae* are opportunistic pathogens, and under certain circumstances where the host has weakened immune defences these bacteria can exploit vulnerabilities and cause disease [31, 38, 39]. The rise of multi-drug resistant (MDR) *Enterobacteriaceae* has been recognised by the Centers for Disease Control and Prevention (CDC). In 2013 the CDC released a report – "Antibiotic Resistance Threats" – which labelled MDR-*Enterobacteriaceae*, including MDR-*K. pneumoniae*, as posing an urgent threat level to human health [11].

K. pneumoniae is found ubiquitously in both the environment, where isolates have been described as being as virulent as human clinical isolates [36, 40, 41], and in humans, where it forms a part of the normal microbiota of the human gastrointestinal (GI) tract. Clinically it is the most significant member of the *Klebsiella* genus, commonly causing nosocomial infections in long-term healthcare settings [42-44]. *K. pneumoniae* is responsible for infections of the urinary tract (UTI), wounds and surgical sites, with the potential to also cause life-threatening bacteraemia and pneumonia [42, 45]. Infections are typically seen in vulnerable patients such as the elderly, immunocompromised, and patients already suffering co-morbidities [45]. Patients in intensive care units and long-term nursing homes, post-surgery patients and those with invasive medical devices are particularly vulnerable [45], whilst exposure to antimicrobial treatment for a prolonged period of time is also a risk factor [46, 47].

K. pneumoniae commonly colonises the GI tract, which forms a reservoir for the spread of *K. pneumoniae* in healthcare environments, and also acts as a potential source of infection for the host further down the line [48]. In addition, studies have found the presence of *K. pneumoniae* on a variety of hospital equipment, ranging from bed rails, mattresses and television monitors, to endotracheal tubes and duodenoscopes [49-54]. This represents a further potential source of infection, and

as a result, adherence to strict infection control and prevention practices is essential to reduce transmission of *K. pneumoniae* and improve infection rates [55].

1.2.1. Severity and implications of *K. pneumoniae* AMR

Treating *K. pneumoniae* infection is difficult and clearance often requires administering antimicrobials [47, 56, 57]. However, a key characteristic of *K. pneumoniae* is its ability to quickly develop resistance *in vivo* to a broad range of antimicrobials [46, 57-59]. This has severe implications for vulnerable patients with weakened immune defences, such as those with underlying illnesses, the elderly or the young. Without the support of effective antimicrobial treatment, infections with MDR *K. pneumoniae* are particularly troublesome [45]. Furthermore, due to the variety of ways in which *K. pneumoniae* can persist in the hospital environment [49-52], there is a real threat of nosocomial outbreaks, with vulnerable patients in particular at risk of hospital-acquired MDR infection [45]. Infections with MDR *K. pneumoniae* dramatically increase the likelihood of death, with mortality rates in excess of 50% [11, 48].

The development of AMR in *K. pneumoniae* and other *Enterobacteriaceae* follows a repetitive pattern: once a new and effective drug is developed, resistance quickly emerges [10, 60]. This is followed by a scramble to identify new drugs or modify existing agents, administer them with initial success, then watch as resistance again develops. This has led to the emergence of numerous *K. pneumoniae* strains with a variety of resistance mechanisms, with the ability to spread globally at a rapid rate.

1.2.2. Mechanisms of acquired resistance in *K. pneumoniae* and global significance

The ability of bacteria to acquire resistance is a common cause of emerging MDR strains. It can arise from spontaneous mutations as a result of selective pressure, where competition for survival under antimicrobial challenge forces only resistant bacteria to survive, or exposure to mutagenizing factors [61]. Mutations can have various ramifications, such as the over-expression of resistance-conferring genes, or alterations to antimicrobial targets which prevent drug recognition and binding [61].

Alternatively, resistance traits can develop through the acquisition of foreign resistance genes via horizontal gene transfer. This can prove particularly challenging to diagnostics and treatment due to the indiscriminate nature of horizontal gene transfer. Resistance genes borne on mobile genetic elements can

pass between bacteria of different species, leading to rapid development of novel forms of resistance [62]. The use of antimicrobials also drives the development of AMR by removing drug-sensitive competitors – both commensal and disease-causing microbes – leaving behind resistant bacteria to reproduce uninhibited [63].

1.2.3. Extended-spectrum β-lactamases

β-lactam drugs, such as penicillins and cephalosporins, are one of the most commonly prescribed groups of antibiotics for treating infections in humans. They work by inhibiting peptidoglycan biosynthesis of the bacterial cell wall, which consequently prevents bacterial growth and leads to cell death [64]. Third-generation cephalosporins (3GCs) were developed in the 1970s and introduced into clinical use in the early 1980s. This was in response to the growing prevalence of TEM-1 and SHV-1 β-lactamases, enzymes capable of hydrolysing and inactivating the existing β-lactam drugs in *E. coli* and *K. pneumoniae*, [65]. These drugs signalled a step forward for treating infections caused by MDR Gram-negative bacteria [65]. However, shortly after their clinical introduction, separate incidents of plasmid-mediated resistance to 3GCs were discovered in Germany and France resulting from extended-spectrum β-lactamases (ESBLs) [66, 67]. These enzymes possessed hydrolysing activity against a broad range of β-lactams, including 3GCs [65]. 3GCs have been shown to select for ESBL-producing *Enterobacteriaceae*, and their use is considered to have played a prominent role in the development of β-lactam resistance [68, 69]. ESBLs can be encoded on mobile plasmids making them easily transferrable between strains, and in the present day, multiple types of ESBL-producing *K. pneumoniae* have been reported globally [65].

1.2.4. Carbapenem-resistant *K. pneumoniae*

Of the β-lactam drugs, carbapenems possess the greatest efficacy against Gram-positive and Gram-negative bacteria and are used to treat serious MDR *K. pneumoniae* and *E. coli* infections [70]. However, the increased use of carbapenems resulted in the emergence of *Klebsiella pneumoniae* carbapenemase (KPC) enzymes. KPCs are β-lactamase enzymes which possess a broad substrate spectrum, capable of hydrolysing most antimicrobial drugs including penicillins, cephalosporins, β-lactamase inhibitors, and carbapenems. KPCs do not however confer resistance to the polymyxins and tigecycline [71-73]. Originally identified in the United States in 1996 as KPC-1 [74], KPC enzymes have now spread globally

among *K. pneumoniae* and other Gram-negative bacteria, typically plasmid-mediated, with a range of KPC variants emerging [75].

Alternative carbapenem resistance mechanisms have also evolved alongside KPCs, such as metallo-β-lactamases (MBLs). These are a diverse group of class B β-lactamases capable of hydrolysing carbapenems [76]. Though MBLs were originally not considered a threat, attitudes towards them changed with the plasmid-mediated spread of IMP- and VIM-type MBLs in the 1990s [64]. More recently, a novel MBL was identified in 2008 when a carbapenem-resistant strain of *K. pneumoniae* was isolated in Sweden from a patient who acquired a UTI after travelling to New Delhi, India [77]. The isolate carried a highly transferrable gene, $bla_{\text{NDM-1}}$, which produces New Delhi metallo-β-lactamase (NDM-1). NDM-1 is resistant to all antimicrobials except fluoroquinolones and colistin [77], and has since been reported worldwide [78-80]. The global dissemination of carbapenemases is a common trait among these enzymes, mainly associated with *K. pneumoniae* [81] and with a propensity for geographical preference e.g. KPC-Americas, NDM-Sub-continent [70, 81, 82]. As a result, the presence of carbapenmases represents a major obstacle to our ability to treat *K. pneumoniae* infections.

1.2.5. Resistance to the last last-lines: the polymyxins

The increasing prevalence of carbapenem-resistant *K. pneumoniae* and subsequent limitations of treatment has led to increased attention on the last-line polymyxin drugs, polymyxin B (PxB) and colistin (PxE) [80]. PxB and colistin are a class of cyclic polypeptide antimicrobials, discovered in 1947 [83] and clinically available in the 1950s [84], which have retained potency against *Pseudomonas aeruginosa* (*P. aeruginosa*), *Acinetobacter* and *Enterobacter* spp., as well as carbapenem-resistant *K. pneumoniae* [4]. The primary target of polymyxin activity in *K. pneumoniae* is the bacterial outer membrane (OM). Interactions between cationic polymyxins and the anionic lipopolysaccharide (LPS) of the OM destabilise OM stability and structure, resulting in increased permeability, cell leakage, and finally cell death [4] (Figure 4).

Previously these antimicrobials had been set aside due to concerns surrounding their use and adverse effects, including nephrotoxicity and neurotoxicity [85, 86]. However, as other agents have become increasingly ineffective, the reliance on these drugs and their clinical use has grown [87, 88]. Indeed, a study by Neuner et

al. [89] looking at the effectiveness of various antimicrobials reported that 86% of carbapenem-resistant *K. pneumoniae* isolates were susceptible to colistin.

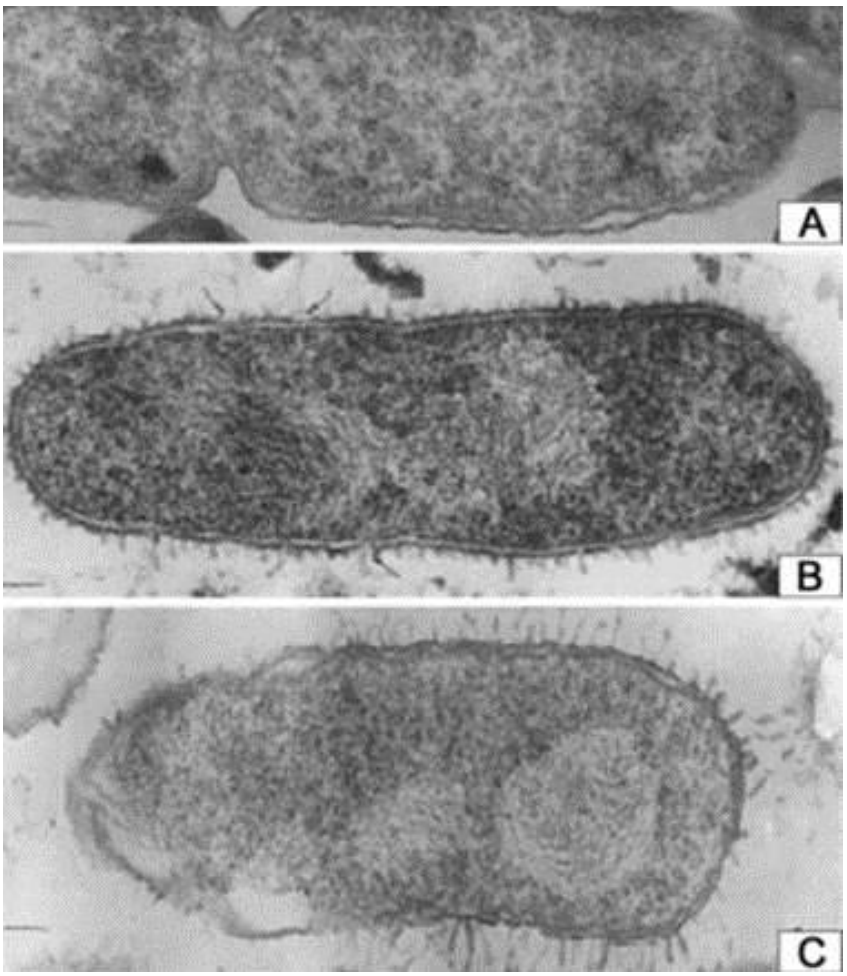

Figure 4. Electron microscopy showing the results of *P. aeruginosa* after exposure to PxB (25 µg/ml) and colistin (250 µg/ml) for 30 min. A) untreated; B) treatment with PxB; C) treatment with colistin. After exposure to polymyxins, alterations and damage to the bacterial cell are clearly visible. *Adapted from Falagas et al., 2005* [4].

Despite this, *K. pneumoniae* isolates have recently emerged exhibiting resistance to all known antimicrobial classes, including the polymyxins. A plasmid-borne polymyxin resistance gene, *mcr-1*, was recently identified among swine in China [90]. This gene was originally identified in *E. coli* but capable of transference and maintenance in *K. pneumoniae* and *P. aeruginosa*. *In vivo*, *mcr-1* expression

decreased the effect of colistin by altering lipid A structure of the LPS. Since its publication, further studies have reported the presence of *mcr-1* in *Enterobacteriaceae* isolates from both humans and animals, indicating its global dissemination [91-93]. Colistin use in intensive farming, with China a notable example, has also seen the emergence of colistin-resistant isolates [94].

Carbapenem-resistant *K. pneumoniae* treated with polymyxin have also been shown to develop resistance *in vivo* [95-98]. Other mechanisms leading to colistin resistance are also emerging, and can be expected to increase with its growing use in treatment and its heavy use in farming [94]. A recent report by the CDC [21] detailed the case of an elderly patient in the USA who died in September 2016 as a result of septic shock. The patient was infected with a strain of carbapenem-resistant *K. pneumoniae* that displayed resistance to all available antimicrobials, including polymyxins. Despite the isolate's resistance to colistin, it tested negative for the *mcr-1* gene, suggesting an alternative mechanism at play.

1.2.6. Intrinsic mechanisms provide alternative paths to resistance

Acquired resistance is clearly a factor in the development of resistance in *K. pneumoniae*, arising from spontaneous genetic mutations and the transfer of genetic material between bacteria via plasmids. However, *K. pneumoniae* already possesses a broad range of intrinsic mechanisms that enable it to resist and persist in harsh conditions.

Intrinsic resistance is the innate ability of a bacteria to tolerate and survive in the face of antimicrobial pressure; resisting the bactericidal or bacteriostatic activity of antimicrobials due to inherent structural and functional processes [61]. This lack of sensitivity to an antimicrobial can result from numerous factors originating from the bacterium. Increased efflux pump activity can export toxic compounds from the cell. Decreased production of OM porins limits the influx of molecules into the bacterium. Additionally, alterations to external surfaces of bacteria prevent microbe recognition by antimicrobials, whilst intrinsically-produced inactivating enzymes are able to degrade and nullify antimicrobials [61]. It is the combination of these intrinsic mechanisms and extrinsic mechanisms which produce the most effective forms of resistance. The comprehensive resistance of *K. pneumoniae* to β-lactam drugs results from various factors, including increased efflux, altered porin activity, and β-lactamase production [64, 70]. Resistance acquired via the transmission of genetic

material is an important factor in AMR. However, when combined with the range of mechanisms already inherently available to the bacterium, the severity of the AMR problem increases.

Global transcription regulators play a major role in mediating intrinsic resistance, capable of activating and inhibiting the expression of a diverse range of physiologically important genes with roles in maintaining cellular homeostasis, biosynthesis, virulence and, significantly, resistance [61]. As a result, the importance of establishing the role of these transcription proteins is critical to the dual phenotypes of antibiotic resistance and virulence.

1.3. RamA: transcription regulator and resistance mediator

RamA belongs to a subfamily of AraC-type transcriptional proteins, which also includes MarA, SoxS, Rob and RarA [99-101]. They are unique among the AraC-type protein family due to their ability to directly bind DNA as monomers [102] and pre-recruit RNA polymerase [103]. They subsequently regulate genes linked to a variety of cellular processes, including virulence and stress response [104], but of particular interest is their link to AMR. These proteins can regulate genes involved in controlling bacterial permeability, alterations to which have been established as a major factor in the development of resistance [105].

The first described MDR-associated AraC-type regulator was the MarA protein in *E. coli*, encoded within the chromosomal multiple-antibiotic-resistance (*mar*) locus [106]. MarA represents the prototype AraC-type resistance regulator, with homologues found among other *Enterobacteriaceae* [107]. MarA controls the expression of genes involved in bacterial permeability [108, 109], and there is considerable overlap between the genes regulated by MarA, and those controlled by SoxS, Rob and RamA [1, 109-113]. *marA* overexpression and its subsequent regulation of permeability genes generates a MDR phenotype, which is similarly conferred by overexpression of *soxS*, *rob* and *ramA* [109, 114]. The resistance phenotype of these regulators is typified by activation of efflux activity, such as upregulation of the AcrAB-TolC efflux pump [115, 116]; reduced influx, for example downregulation of the OM porin OmpF [117, 118]; and alterations to lipid A and LPS of the bacterial OM [1, 119].

AMR is a multifactorial process which can arise from single, separate events or result from a combination of mechanisms [61, 120]. Consequently, resistant *Enterobacteriaceae* isolates can demonstrate similar resistance phenotypes to a variety of antibiotics, but which develop from separate mechanisms. O'Regan et al. [121] reported that *acrB* overexpression, downregulated *ompF*, and LPS alterations resulting from overexpressed *soxS*, *marA* and *ramA* all contributed to resistance in different *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) isolates. This indicates a complex bacterial system where numerous compensatory resistance mechanisms are able to promote survival under harsh conditions. Furthermore, it is proposed that MarA, SoxS, Rob and RamA are essential components of early stage resistance development because they promote bacterial survival in the face of host

immunity or antimicrobial challenge. This provides sufficient time under selective pressure for the bacteria to develop or acquire further beneficial resistance mutations [1, 122].

MarA, SoxS, Rob and RamA have all been shown to substantially contribute to the development of resistance to a broad range of antibiotics; however, their regulation of many resistance genes, such as the *acrAB* efflux pump or *ompF* porin, generally overlaps [1, 109, 110, 112, 123-125]. Therefore, what is unclear is the relative importance of each AraC-type regulator and what they contribute to AMR: is overlapping regulation a protective back-up mechanism in the event that one regulator is inactivated, or do specific regulators have more significant roles than others in AMR in a species- or gene-dependent manner? *E. coli*, for example, is able to resist antimicrobial challenge due to overexpression of *marA*, *rob* or *soxS* [109, 124, 126]. However, RamA, which is not genomically encoded in *E. coli* [127], has been strongly linked to MDR in both *K. pneumoniae* and *Salmonella* spp. [125, 128-130], and can activate expression of MarA-, SoxS-, and Rob-regulated resistance genes independently of these regulators [128, 131]. Furthermore, expression of *K. pneumoniae ramA* in *E. coli* is able to confer an identical MDR phenotype to that found in *K. pneumoniae* [101]. Contrastingly, it has been reported that inactivation of *ramA* does not increase antimicrobial susceptibility in *S.* Typhimurium [132]. However, the authors fail to take into account that the MDR phenotype is conferred by *ramA* overexpression, as opposed to normal *ramA* levels [1, 127], indicating that the proposed role of *ramA* from this study was not fully investigated. As a result, elucidating the significance of specific regulators in specific species is important to understanding resistance determinants and how best to counter them. Due to the emergence of *K. pneumoniae* as a MDR pathogen of increasing global importance, efforts to better understand the intrinsic mechanisms that contribute to bacterial AMR are essential.

1.3.1. Regulation of *ramA*

RamA is encoded by the *ramA* gene which, besides *Klebsiella*, is also found in other *Enterobacteriaceae* spp. including *Enterobacter*, *Citrobacter* and *Salmonella* [127, 133, 134]. The *ram* locus is conserved in all of these organisms bar *S.* Typhimurium, which lacks the upstream *romA* gene, proposed to encode a MBL [1]. Under normal conditions, RamR, a TetR-type family regulator encoded by the *ramR* gene, represses expression of the *romA-ramA* genes. Basal *ramA* levels are maintained

due to the interaction of the small regulatory RNA sRamA5, a cleaved by-product of the *romA* transcript, with the repressor RamR, thus providing derepression to allow the basal levels of *ramA* transcription. Whilst RamR has a higher binding affinity, the competition of sRamA5 is sufficient to help maintain basal *ramA* expression [1]. Inactivating mutations to the *ramR* gene [135-137], and inhibitory ligand-mediated interactions with RamR [138, 139], result in increased *ramA* expression [138, 140], which subsequently confers a MDR phenotype [1, 127, 141, 142] (Figure 5).

Inactivating *ramR* mutations leading to *ramA* overexpression have been reported as a result of tigecycline exposure [135, 143], which results in reduced susceptibility to this agent. Of interest, clinical isolates that pre-date the use of tigecycline also exhibit *ramA* overexpression, indicating the ability of other antibiotics to select for this change [134]. This implicates RamA as an important mechanism which *K. pneumoniae* can utilise to resist a range of antimicrobials. The development of tigecycline resistance is important because tigecycline is an antimicrobial of increasing interest for the treatment of MDR *K. pneumoniae* [144-146]. The role of RamA in conferring resistance to this agent is notable as it highlights a major role for this intrinsic mechanism in mediating effective resistance to a key antibiotic.

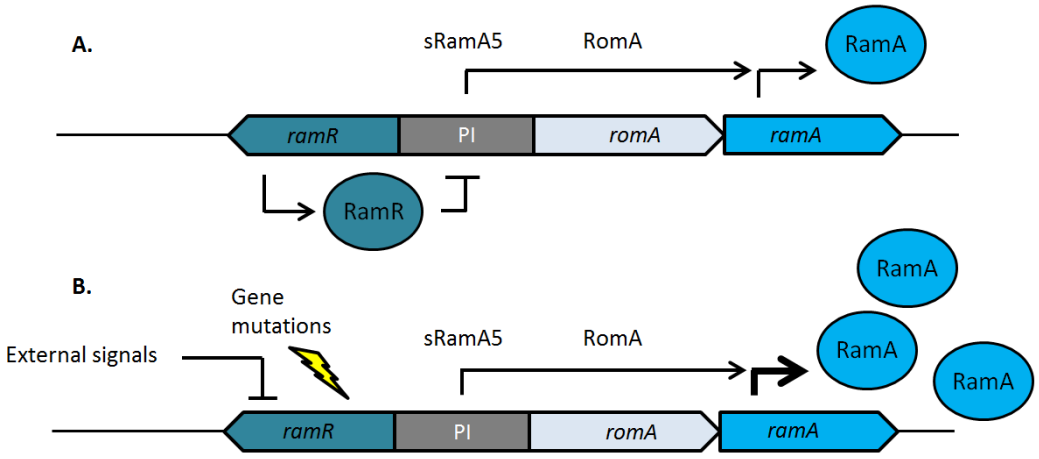

Figure 5. Organisation of the *ram* locus in *K. pneumoniae*. A) Under normal conditions, expression of *ramR* results in repression of *romA-ramA*. The small regulatory RNA, sRamA5, interacts with RamR and competitively binds for the PI promoter, thereby maintaining basal levels of *ramA* expression. Inactivating mutations to *ramR* or repressive external signals allow unregulated expression of *ramA. Adapted from de Majumdar et al, 2015.* [1].

1.3.2. The RamA regulon in *K. pneumoniae* and its role in resistance

K. pneumoniae is a pathogen of growing importance in AMR due to the increasing prevalence of MDR strains worldwide. While several studies have reported the effects of RamA regulation in *S*. Typhimurium, until recently the scope of the RamA regulon in *K. pneumoniae* had not been addressed. In *S.* Typhimurium, RamA has been demonstrated to play an important role in bacterial resistance and survival after antimicrobial challenge [125, 140]. Studies have found that *ramA* gene disruption leads to decreased survival within macrophages [140]. Conversely, *ramA* overexpression leads to increased activation of MDR genes, including the efflux pump genes *acrAB*, *acrEF*, and *tolC*, and decreased expression of the porin gene *ompF*, resulting in decreased antimicrobial susceptibility [125, 140]. Overexpression also promotes immune evasion via reduced adhesion to, and improved survival within, macrophages [125, 140]. These findings indicate the importance of RamA regulation to the *Salmonella* AMR phenotype.

The recent study by Majumdar et al. [1] determined the RamA regulon in *K. pneumoniae* Ecl8, revealing a total of 103 differentially expressed genes in the presence of increased *ramA* levels, with roles in a variety of important cellular processes. Importantly, when *ramA* is overexpressed, genes associated with bacterial permeability, such as efflux pump and porin genes, are most affected. Transcriptome and qPCR analyses of *ramA* overexpression showed overlaps with the regulons of MarA, SoxS, and Rob in different *Enterobacteriaceae* spp., with regards an upregulation of efflux pump gene *acrAB* [1, 108, 125, 140]. Additional OM-linked genes were shown to be upregulated: *tolC*, a component of the AcrAB efflux pump complex; the efflux-related operons *oqxAB* and *yrbB-F; lpxL-2*, encoding a lipid A biosynthesis lauroyl acyltransferase; and *lpxO*, another gene in the lipid A biosynthesis pathway encoding a dioxygenase [1]. The OM porin *ompF*, on the other hand, was downregulated. Furthermore, electrophoretic gel shift mobility assays (EMSAs) and transcription *in vitro* (IVT) assays confirmed that RamA is able to directly bind and activate the promoters of *acrAB*, *yrbF*, *lpxL-2*, and *lpxO* [1].

1.3.3. Significance of differentially modulated AcrAB, TolC and OmpF in AMR

Efflux pumps play an important role in bacterial AMR; increasing the expulsion of toxic compounds from within the cell, preventing intracellular accumulation and negating lethal effects, and consequently promoting bacterial survival (Figure 6). Efflux pumps are effective due to the dynamic ways in which they confer resistance;

inherently resistant to a broad spectrum of antimicrobial classes or with high substrate specificity to specific drugs. Alternatively, resistance can be conferred by upregulated efflux as a result of gene mutation or mediated by global regulators [122]. In *Enterobacteriaceae*, numerous studies have demonstrated that overexpression of either *acrAB* or *tolC* genes, encoding the AcrAB-TolC efflux pump, often results from increased expression of the AraC-type regulators MarA, SoxS and RamA [1, 99, 113, 126]. Consequently, this results in reduced susceptibility to a broad range of antimicrobials, as well as decreasing bacterial virulence [122, 147-149]. Previous studies have also established the importance of a functional AcrAB efflux pump in the resistance phenotype; in its absence, overexpression of MarA, RarA and RamA fail to confer resistance to a variety of antimicrobials [130, 150].

Porins also play an important role in reducing antimicrobial susceptibility. These OM proteins, such as OmpF, participate in the diffusion of antimicrobials into the bacterial cell [105, 151]. Reducing the expression and activity of porins prevents the transport and build-up of antimicrobials and, combined with increased efflux activity, limits bacterial permeability sufficiently to reduce antimicrobial susceptibility [152-155] (Figure 6). Expression of *ompF* is controlled by MarA, SoxS and RamA [1, 156]. When these regulators are overexpressed, they activate expression of *micF* [157], an antisense RNA which subsequently decreases expression of *ompF*, reducing efflux and conferring reduced antimicrobial susceptibility [158-160].

Reduced bacterial permeability via increased efflux and reduced influx is an important intrinsic mechanism that *K. pneumoniae* can utilise in order to lower antimicrobial susceptibility. RamA is therefore a significant factor in conferring bacterial AMR due to its ability to upregulate and downregulate efflux and porin genes respectively [1]. However, RamA has also been shown to activate expression of the lipid A biosynthesis genes *lpxL-2* and *lpxO* [1]. Lipid A is an important component of the Gram-negative OM, and RamA-mediated modifications to lipid A synthesis may be an important factor in preventing antimicrobial targeting and killing of bacteria. This indicates another potentially significant role that this intrinsic regulator has in conferring an AMR phenotype to *K. pneumoniae*.

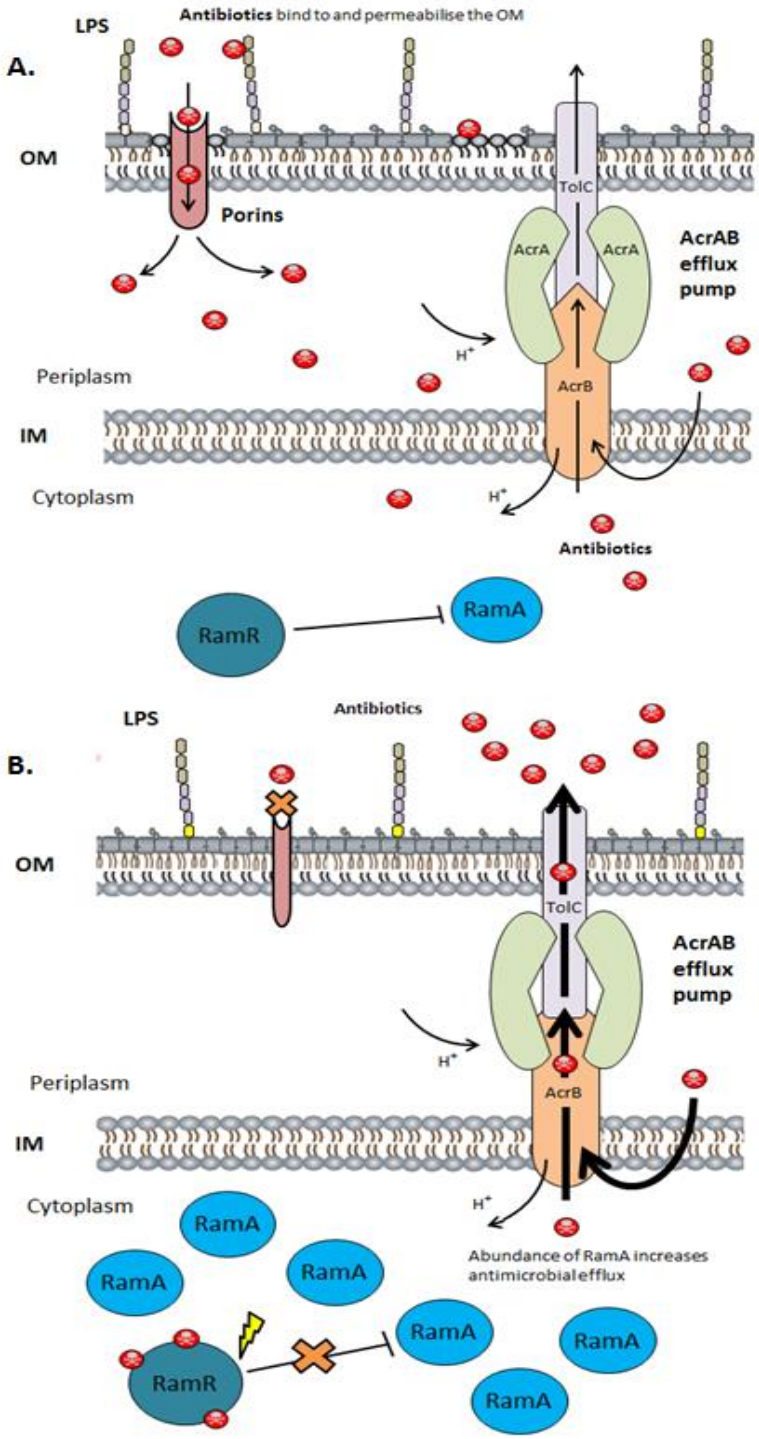

Figure 6. Effect of RamA upregulation on bacterial permeability and antimicrobial susceptibility. A) Porins such as OmpF control import of molecules into the cell via diffusion, including antimicrobials, while efflux pumps are responsible for exporting foreign and harmful compounds; B) when the *ramA* repressor, RamR, is rendered inactive, *ramA* is upregulated. Subsequently, efflux activity increases and porin-mediated import decreases. These changes contribute to antimicrobial resistance. *Adapted from Malinverni et al., 2009* [5].

1.3.4. Lipid A of the Gram-negative outer membrane

Controlling bacterial permeability is essential to the organism's survival, and the Gram-negative bacterial OM plays a central role. It provides a vital protective barrier, acting as an obstacle in the path of macromolecules or hydrophobic antimicrobials due to its hydrophobic lipid bilayer composition [161]. Despite this, it succeeds in allowing uninterrupted import and export of materials essential for bacterial homeostasis, courtesy of interspersed OM porin proteins and efflux pumps. Lipopolysaccharide (LPS) is a major component of Gram-negative bacterial OMs and is essential for bacterial growth [105, 162-164]. It consists of three parts: a highly variable O-antigen and a core oligosaccharide region, which is anchored to the OM by lipid A, a phosphorylated disaccharide possessing hydrophobic fatty acid chains. LPS functions as an externally-facing protective barrier due to its hydrophobic nature, but also functions as an endotoxin, with lipid A the primary immunostimulant behind endotoxic activity [105].

Lipid A forms the outermost layer of the Gram-negative OM, and subsequently has an important role in host-pathogen and host-drug interactions. Innate immunity is the host's first line of defence against infection; its responsibility is to recognise pathogens and clear them quickly and effectively. Toll-like receptor 4 (TLR4) is a member of the TLR family of pattern-recognition receptors (PRRs) which differentiate between the host and threats by recognising pathogen-associated molecular patterns (PAMPs). The lipid A of LPS is one such PAMP to which TLR4 has specificity [165-169]. TLR4 recognition of LPS induces a signalling cascade which stimulates the production of pro-inflammatory cytokines [165], induces macrophages to produce inflammation mediators such as TNF-α and IL-1β [170, 171], and is also able to activate co-stimulatory molecules that trigger adaptive immunity [172, 173]. Whilst these responses are fundamental to innate immunity and beneficial to clearing infection from the host, when overproduced, for example during sepsis, the lipid A stimuli elicit an overwhelming inflammation response which causes harmful damage to the host, potentially resulting in septic shock [174, 175].

Lipid A and the LPS core region carry several anionic groups, resulting in a net negative charge which favours cation binding. This allows for strong lateral interactions between neighbouring anionic LPS molecules and divalent cations in the OM, presenting an effective barrier projecting from the OM surface [161]. However, while the anionic nature of LPS is fundamental to its ability to form a

hydrophobic barrier, it also makes LPS a prime recognition and binding candidate for cationic antimicrobial peptides (CAMPs), including polymyxin drugs and CAMPs generated by the host immune response [105].

The polymyxins PxB and colistin are last-line CAMPs whose therapeutic use in MDR-*K. pneumoniae* infections has seen a resurgence alongside the rise of strains resistant to all other available agents [4, 80]. The mechanism behind polymyxin bactericidal activity is two-fold: initially, due to a higher binding affinity, the cationic polymyxins competitively displace the divalent cations of the OM to bind anionic lipid A, destabilising and permeabilising the OM; this in turn allows access to the bacterial inner membrane (IM), where subsequent permeabilisation and cytoplasmic leakage lead to cell death [105, 161, 176]. When used singularly, polymyxins possess effective bactericidal activity [176, 177], though resistance to monotherapy has been shown to quickly develop [178, 179]. However, combination therapy with additional drugs holds great promise. Because even low polymyxin concentrations are sufficient to permeabilise the OM, polymyxin is able to facilitate the passage of hydrophobic antimicrobials to which the OM would otherwise be resistant, dramatically increasing sensitivity [161, 180-182].

1.3.5. The role of lipid A modifications in contributing to polymyxin resistance and promoting bacterial survival

An essential step in polymyxin-mediated bacterial killing is the electrostatic interaction between the CAMP and the anionic lipid A of LPS. As a result, Gram-negative species have developed various mechanisms to counter the effects of CAMPs, many of which rely on modifications to lipid A [176] (Table 1). Canonical lipid A is expressed by the *E. coli* K12 strain, and lipid A biosynthesis genes are conserved amongst Gram-negative bacteria [183]. However, lipid A structure is heterogeneous, characterised by varying fatty acid chains, different chemical moieties, and diverse acylation and phosphate patterns, all of which can contribute to resistance and persistence [183, 184]. The most common lipid A modifications are achieved through the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) and phosphoethanolamine (PEtN) [185-190]. These lipid A modifications have the effect of lowering the net negative charge of LPS [176], potentially decreasing the natural repulsion between neighbouring anionic LPS molecules and producing a more closely packed layer of LPS [105]. As a result of the decreased negative charge of

LPS, initial polymyxin-pathogen interactions are limited, thereby reducing polymyxin-mediated permeabilisation of the OM and preventing cell death [176, 177].

In *Klebsiella* spp., lipid A modifications are controlled by the auto-regulating PhoP-PhoQ (PhoPQ) two component regulatory system [177]. This two-component system (TCS) is also found in other Gram-negative bacteria including *Salmonella* spp., *E. coli*, and *P. aeruginosa*. TCSs allow bacteria to regulate gene expression and mediate adaptive changes in response to environmental stimuli. The membrane-localised PhoQ sensor protein responds to environmental low divalent cation concentrations, such as Mg^{2+} starvation or the presence of CAMPs, by auto-phosphorylating [191, 192]. It then activates the cytosolic PhoP DNA-binding response regulator via a transphosphorylation reaction which leads to activation or repression of target genes [193-195]. PhoPQ also regulates the PmrA-PmrB (PmrAB) two-component system, creating a signal cascade which in turn modulates expression of genes involved in lipid A modification. PhoPQ and PmrAB-regulated genes involved in lipid A modifications include *pmrC,* responsible for PEtN synthesis [196]. Also regulated are *pmrE* and *pmrH*, which encode enzymes responsible for synthesising and attaching L-Ara4N to lipid A, neutralising the negative charge of LPS and generating a phenotype associated with colistin resistance [196-199]. PmrAB is also able to regulate lipid A modifying genes independently of PhoPQ, where the PmrB sensor autophosphorylates in response to low pH or environmental levels of Fe^{3+} [188, 200], before activating the response regulator PmrA [201].

Mutations in PhoPQ and PmrAB resulting in their constitutive expression lead to resistance to polymyxins as well as a range of structurally distinct CAMPs [177, 202]. Unsurprisingly, inactivating mutations have the opposite effect whereby susceptibility to CAMPs is dramatically increased [202-204]. In particular, constitutive activation of PhoP as a result of *phoQ* mutation results in reduced susceptibility to polymyxins and CAMPs [198, 203, 204], whilst when PhoP is inactivated, mutants become hyper-sensitive [194, 202]. PhoPQ is also negatively regulated by the *mgrB* gene, inactivating mutations of which give rise to colistin resistance in MDR *K. pneumoniae* [205, 206].

An additional way in which lipid A modifications are proposed to contribute to bacterial survival is via evasion of innate immunity. These modifications may result in the failure of PRRs of the immune response to recognise bacterial lipid A and

detect the presence of the bacterium. TLR4 has difficulty recognising PhoPQ-regulated lipid A [207] with PhoPQ-mediated lipid A deacylation and palmitoylation implicated [208]. This essentially hides the pathogen from the host's immune system, and in *K. pneumoniae* has been shown to limit activation of inflammatory responses [183]. As a result, the role of these regulatory TCSs and lipid A modifications are crucial to immune evasion and to the development of CAMP resistance in various Gram-negative bacteria, including *K. pneumoniae*.

Table 1. Polymyxin resistance mechanisms of Gram-negative bacteria.

Bacterium	**Examples of resistance mechanisms**
P. aeruginosa	PmrA/PmrB-mediated lipid A modifications with l-Ara4N
S. Typhimurium	PmrA/PmrB-mediated lipid A modification with both l-Ara4N and PEtN The gene *mig-14* is required for resistance but does not involve LPS modification
E. coli	PmrA/PmrB-mediated lipid A modification with both l-Ara4N and PEtN
K. pneumoniae	Increased production of capsule polysaccharide.
Vibrio cholerae	Presence of OM protein OmpU regulated by ToxR

Table 1. Many resistance mechanisms that Gram-negative bacteria have developed in response to CAMPs involve modifications to lipid A of the LPS. This affects overall net charge, thereby impacting on and limiting the initial electrostatic interaction between CAMPs and the bacterial OM. *Adapted from Zavascki et al., 2007* [176].

1.3.6. LpxO modifies lipid A, contributing to AMR

The *lpxO* gene has orthologues in various Gram-negative bacteria, including *Salmonella* and *Klebsiella* spp.. It encodes LpxO, an IM-localised dioxygenase with

an active site predicted to face into the cytoplasm [191]. Conditional on bacterial growth in O_2, LpxO is involved in the biosynthesis of the 2-hydroxymyristate modifications of specific lipid A acyl chains [184, 209]. This moiety is proposed to enhance hydrogen bonding between lipid As, stabilising the OM and increasing its impermeability [191]. Expression of *Salmonella lpxO* in *E. coli* K-12, which does not naturally possess the gene, introduces the ability to modify lipid A with 2-hydroxymyristate in an O_2-dependent manner [209]. In *S.* Typhimurium, *lpxO* inactivation results in the abolition of 2-hydroxymyristate-modified lipid A [191].

In *S*. Typhimurium, *lpxO* expression is in part regulated by PhoPQ [207, 209, 210]. PhoPQ is essential for bacterial resistance to CAMPs and can also be induced by CAMPs, leading to PhoPQ-mediated activation of virulence and resistance genes, including lipid A modifiers [211, 212]. Llobet et al. [211] reported that pre-treatment of *K. pneumoniae* with PxB produced lipid A with modifications that contributed to cross-resistance to host immune CAMPs. Among these lipid A modifications, species were recovered that possessed hydroxymyristate modifications which correspond to the activity of LpxO in *S*. Typhimurium [211]. This indicated a possible *K. pneumoniae* LpxO orthologue and a potential role for this enzyme in CAMP-induced resistance [211]. The presence and activity of *K. pneumoniae* LpxO was later confirmed in a subsequent study by Llobet et al. [183]. They reported that *K. pneumoniae* is able to modify its lipid A in a tissue-dependent manner. Lipid A recovered from the lungs of a *K. pneumoniae* murine infection model demonstrated a lipid A moiety consistent with the PhoPQ-regulated, LpxO-dependent 2-hydroxymyristate modification [183]. This lipid A modification reduced susceptibility to CAMPs and inhibited activation of inflammation responses. The authors suggest that the LpxO-mediated, tissue-dependent lipid A modification recovered from the lung was due to induction by CAMPs, likely present in the environment at sub-inhibitory concentrations. This is consistent with their findings that the same *in vivo* lipid A pattern was inducible by colistin, while existing colistin-resistant isolates already expressed it [183]. This indicates the ability of *K. pneumoniae* to modulate gene expression to promote survival, and the potential importance of LpxO in mediating immune evasion and resistance to CAMPs.

It has been reported in *S.* Typhimurium that *lpxO* is able to remain active in the absence of PhoPQ regulation [213-215]. Recently, Majumdar et al. [1] established the role of RamA as a regulator of *lpxO* in *K. pneumoniae*. Overexpression of *ramA*

led to increased expression of *lpxO* apparently independent of PhoPQ, as no concurrent increase in *phoP* expression was observed. They also demonstrated that *ramA* overexpression in *K. pneumoniae* leads to reduced susceptibility to PxB, colistin, and the human CAMP LL-37 [1]. Overexpression of *ramA* upregulates efflux pump genes *acrAB* and *tolC*, and downregulates the porin gene *ompF* [1], the effects of which are associated with conferring increased resistance to antimicrobials [122, 148, 152]. In *K. pneumonia*e, RamA-mediated decreased antimicrobial susceptibility to most drugs is reliant on the presence of a functional AcrAB efflux pump [1]. AcrAB in *K. pneumoniae* has also been shown to have a possible role in virulence and CAMP resistance; where *acrAB* knockout mutants demonstrate a reduced ability to cause pneumonia in mice, and increased susceptibility to CAMPs from the human lung [216]. However, resistance to polymyxins and host immune peptides is often attributed to lipid A modifications that affect the ability of CAMPs to bind and permeabilise the bacterial OM [176, 177]. In addition, in *K. pneumoniae ramA* overexpression protects the bacterium from macrophage uptake, consistent with previous reports whereby modified lipid A contributes to immune evasion [183, 207, 208]. As a result, the contributions of the various resistance associated genes – *lpxO*, *acrAB*, *tolC*, and *ompF* – to the RamA-mediated AMR phenotype are unknown. Regulation of bacterial permeability or lipid A modifications may play a role in individually reducing antimicrobial susceptibility, or may work in concert to promote higher levels of resistance (Figure 6).

There is a scarcity of literature concerning *lpxO*, relative to the well-characterised efflux pump and porin genes. The role of LpxO in conferring lipid A-mediated CAMP resistance and promoting immune evasion in *K. pneumoniae* still needs exploration, and the specific factors behind its regulation are unclear. MDR *K. pneumoniae* is on the rise, and resistance to even last-line polymyxin drugs is beginning to emerge. Understanding how polymyxin resistance develops is crucial to efforts to combat the increasing threat of this global pathogen. Defining the regulation of *lpxO* and its contribution to the AMR phenotype in *K. pneumoniae* is an important step in elucidating the mechanisms that this bacterium employs to resist and persist in the face of antimicrobial challenge.

2. Hypothesis

It is established that *K. pneumoniae* RamA contributes to changes in antimicrobial susceptibility to a broad range of agents through the perturbation of microbial permeability, via altered efflux and influx. Increased *ramA* expression results in decreased susceptibility to the last line polymyxin antimicrobials, PxB and colistin [1]. It has also been reported that the *lpxO* gene is upregulated in response to increased *ramA* expression [1]. The function of LpxO is to modify bacterial lipid A in a way that stabilises and impermeabilises the bacterial OM [191], and *lpxO* expression confers a similar phenotype of reduced CAMP susceptibility and increased virulence to that observed with *ramA* overexpression in *K. pneumoniae* [1, 183, 211]. As a result, how RamA-mediated changes to microbial permeability and modifications to lipid A contribute to and control polymyxin susceptibility is still uncertain. Given that the target of CAMPs and polymyxins is the bacterial OM, we hypothesise that RamA-mediated regulation of lipid A is a key factor in conferring resistance to polymyxins and CAMPs.

The relevance of RamA regulation is reflected in the potential implications these changes will have on both antimicrobial resistance and virulence. The recent and rapid emergence of colistin-resistant *K. pneumoniae* isolates has made it critical to understand the basis of transmission and dissemination of these isolates. Given the increasing reliance on colistin to treat MDR *K. pneumoniae* infections, it is essential to determine intrinsic mechanisms that contribute to the development of resistance.

3. Aims

1. Characterise the impact of *lpxO* inactivation on susceptibility to the last-line polymyxins, PxB and colistin and its regulation by RamA. LpxO-mediated lipid A modifications have been reported to be important for resistance to CAMPs and colistin in *K. pneumoniae* [183, 206], whilst *ramA* overexpression is linked to a similar phenotype, as well as to increased expression of *lpxO* [1]. We planned to generate *lpxO* knockout mutants in *K. pneumoniae* w/t and *ramA* overexpressing strains. This would allow us to assess if loss of LpxO-mediated lipid A modifications affects the RamA-mediated MDR phenotype, and thereby examine whether RamA regulation of *lpxO* is important for polymyxin resistance.

2. Determine the role of PhoPQ in the RamA-mediated AMR phenotype and regulation of *lpxO*. RamA and PhoPQ have both been linked to the regulation of *lpxO* in *K. pneumoniae* [1, 183, 211]. We sought to determine whether PhoPQ contributes to the AMR phenotype attributed to *ramA* overexpression [1] through regulation of *lpxO*. We aimed to achieve this by generating *phoPQ* knockout mutants in w/t and *ramA* overexpressing backgrounds and comparing polymyxin susceptibility to corresponding *lpxO* knockouts.

3. Define the contribution of *lpxO*, *ramA* and *phoPQ* overexpression to polymyxin and tigecycline resistance. Overexpression of *ramA* is associated with tigecycline and polymyxin resistance through regulation of bacterial permeability genes [1, 125, 140], whilst *phoPQ* is associated with resistance to CAMPs and polymyxins via regulation of lipid A modifying enzymes [177, 183, 196-199, 211]. LpxO-mediated lipid A modifications have been shown to be important for CAMP and colistin resistance, and *lpxO* is regulated by RamA and PhoPQ [1, 183, 211]. Therefore we aimed to determine and compare the effects of *lpxO*, *ramA* and *phoPQ* overexpression in *K. pneumoniae* on susceptibility to tigecycline, PxB and colistin. We generated plasmid constructs overexpressing these genes and introduced them into w/t and *ramA* overexpressing strains, comparing susceptibility phenotypes with *K. pneumoniae* strains possessing knockouts of RamA-regulated permeability genes.

4. Materials and Methods

4.1. Bacterial growth conditions

Generally, bacteria were grown from frozen -80°C stocks on Luria-Bertani (LB) agar (1.5% bacteriological agar, 1% tryptone, 1% NaCl, and 0.7% yeast extract) at 37°C. The resultant single colonies were used to inoculate 5 ml of LB broth (Formedium LB broth Lennox) with/without antibiotics for selection and shaken at 37°C or 30C overnight.

4.2. Cell lysate DNA (PCR template)

4.2.1. Broth culture

Bacteria were inoculated in LB broth and left to shake overnight, with antibiotics and temperature dependent on the strain. Overnight culture was decanted into a micro-centrifuge tube and centrifuged for 3 min at maximum (max) speed. Supernatant (SN) was removed and the pellet re-suspended in sterile distilled H_2O (sdH_2O); this was again centrifuged for 3 min at max speed, SN removed, and the pellet re-suspended in sdH_2O. The sample was placed in a heating block at 95°C for 10 min, then immediately chilled on ice for 5 min. The sample was centrifuged for 3 min at max speed, with the resulting DNA-containing SN removed for immediate use or storage at -20°C.

4.2.2. Agar plate colony

A single colony was picked from an agar plate and re-suspended in 100 µl sdH_2O. The sample was then heated at 95°C for 10 min, before being chilled on ice for 10 min. After centrifugation for 3 min at max speed, the DNA-containing SN was removed and stored at -20°C.

4.3. Polymerase Chain Reaction (PCR)

For PCR screening, Bioline MyTaq^{TM} DNA Polymerase was used as per the manufacturer's protocol. For amplification of DNA to be subsequently used in downstream reactions, NEB Q5® High-Fidelity Polymerase was used.

4.4. PCR and gel purification

PCR purification was performed using the ThermoFisher GeneJET PCR Purification Kit, as per the manufacturer's protocol. For gel purifications, the PCR purification protocol was followed, with the additional step of melting a 1:1 mixture of excised DNA-containing gel and binding buffer at 55°C for 10 min, prior to using the spin column.

4.5. Genomic DNA purification

Genomic DNA purification was performed using the Promega Wizard Genomic DNA Purification Kit, as per the manufacturer's protocol.

4.6. Gram-negative plasmid extraction

Bacterial cultures grown overnight were decanted into micro-centrifuge tubes which were then centrifuged for 3 min at max speed, with the SN discarded. The bacterial pellet was re-suspended in 200 µl of Solution 1 (50 mM Tris-Cl pH 8.0, 10 mM EDTA, RNAse A 0.1 g/L) by vortexing, followed by the addition of 200 µl Solution 2 (200 mM NaOH, 1% SDS (sodium dodecyl sulfate)) with gentle mixing by inversion in order to lyse the cells. Two hundred microlitres of Solution 3 pH 5.5 (3 M potassium, 5 M acetate) was added, which created a precipitate of cellular components and DNA. The samples were chilled on ice for 5 min, and then centrifuged for 10 min at max speed. The resulting DNA-containing SN was transferred to micro-centrifuge tubes containing 450 µl isopropanol and mixed thoroughly by inversion, after which they were left to stand for 5 min and then centrifuged for 10 min at max speed. SN was discarded leaving only the DNA pellet, which was gently washed with 70% ethanol. The ethanol was removed and the pellet air-dried for 10-15 min to evaporate the ethanol. The DNA pellet was finally re-suspended in 50 µl dH_2O and stored at -20°C.

4.7. DNA ligation reactions

Ligation reactions used a set formula, detailed below, to calculate the amount of insert required for the concentration of vector used. Ligation reactions were incubated overnight at 4°C, before transformation into calcium-competent cells the following day.

$$\frac{vector\ conc. \times insert\ size\ (kb)}{vector\ size\ (kb)} \times \frac{3}{1}$$

4.8. Calcium-competent cells (CCs)

Fifty millilitres of LB broth was inoculated with overnight culture with/without antibiotics for selection, which was then grown to $O.D._{600}$ 0.6. The culture was centrifuged for 15 min at 4°C, 4,000 rpm, and the SN discarded. The pellet was re-suspended in 50 ml of ice-cold 0.1 M $CaCl_2$, re-centrifuged, and SN discarded. This was repeated twice more, with 25 ml and 5 ml of ice-cold 0.1 M $CaCl_2$. Finally, the suspension was centrifuged for 15 min at 4°C, 4,000 rpm, with SN discarded and the

pellet re-suspended in 1 ml of ice-cold 0.1 M $CaCl_2$ and 110 µl of 90% glycerol. CCs were stored at -80°C and validated before use. Validation involved inoculating agar plates containing antibiotics to which CCs should be sensitive, to confirm that CCs were not contaminated.

4.9. Heat-Shock transformation

Ligation reactions were mixed with 100 µl of CC-DH10β cells and incubated on ice. After 30 min, the reaction was placed in a 42°C water bath for 45 sec, before being returned immediately to ice for 1 min. One millilitre LB broth was added and the reaction incubated for 1.5 hours at 37°C. After incubation, the reaction was spun down, SN discarded and the pellet re-suspended in 100 µl LB broth. Ten and ninety microlitres were plated on separate LB agar (with/without antibiotics for selection) and grown overnight at 37°C.

4.10. Electro-competent cells (ECs)

Overnight culture was used to inoculate 50 ml LB broth 1:100, with/without antibiotics for selection, and grown to $O.D._{600}$ 0.6. The culture was centrifuged for 15 min at 4°C, 4,000 rpm, and the SN discarded. The pellet was re-suspended in 50 ml ice-cold 10% glycerol, re-centrifuged, and the SN discarded. This was repeated twice more, before a final re-suspension of the pellet in 500 µl ice-cold 10% glycerol. Washing steps are intended to remove salts from cells in order to protect them from electroporation; addition of glycerol is for protection during freezing. Forty microlitre volumes of this suspension were aliquoted into micro-centrifuge tubes for immediate use or storage at -80°C, with one sample used for validation (see CC cells).

4.11. Electroporation transformation

Electroporation cuvettes were chilled on ice for 15 min before EC cells were added. For each sample of cells to be electroporated with plasmid, a control sample was also set up. These controls were treated in the same way, minus the addition of plasmid. The amount of plasmid used varied depending on its purpose; 250 – 1000 ng was added to cells which were then mixed. Each sample was then pulsed with 1800 V, following which 1 ml of LB broth was immediately added and mixed. The samples were shaken for 1 hour, after which they were centrifuged for 3 min at max speed, SN discarded, and re-suspended in 100 µl LB broth. Ten and ninety microlitres of each sample were aliquoted onto separate LB agar plates, with the

necessary antibiotic selection. These plates were left to grow for 24 to 48 hours at either 30 or 37C.

4.12. Gene exchange protocol

The exchange protocol was performed as previously described by Merlin et al. [217]. The method requires the replacement of the target gene with a FRT (Flp recognition target)-flanked cassette containing the kanamycin resistance (Km^R)-encoding *aph* gene. This cassette resides within a temperature-sensitive pTOF plasmid which also possesses a chloramphenicol resistance (Cm^R)-encoding gene. The process comprises three steps: *integration* of the Km cassette into the bacteria by homologous recombination; *purification* of the bacterial population, where the Km cassette is integrated into the bacterial chromosome and transformants cured of the pTOF plasmid; and *generation* of the mutant strains, where colonies displaying appropriate phenotypic and genotypic characteristics are frozen and stored for future work. The process provides simple selection for correct, gene-deleted transformants via acquisition of Km^R and loss of Cm^R. Furthermore, due to the FRT sites flanking the cassette, the cassette can be excised by a Flp recombinase-producing plasmid at a later stage.

The Km cassette-bearing pTOF plasmid was electroporated (250 ng) into the appropriate strain for target gene replacement and left to grow overnight at 30°C on LB agar/Km plates. Single colonies were picked and grown in LB broth/Km overnight in a 30°C shaker. The culture was serially diluted 10-fold, spread on LB agar/Km and incubated at 30°C and 42°C. The higher temperature of 42°C prevents the plasmid from autonomously replicating; the large single colonies that result from these conditions indicate that the cassette has been forced to integrate with the chromosome and are therefore viable for continuing the protocol. At 30°C, the control temperature permissive for plasmid replication, no large colonies should be present and colonies will be indistinguishable from each other. Large single colonies from 42°C plates were picked and purified by streaking on LB agar/Km plates and incubating overnight at 42°C. This step was repeated to ensure transformants were cured of plasmid. Following the second 42°C incubation, single colonies from each streaking were pooled in LB broth/Km and shaken overnight at 30°C. LB broth was then inoculated 1:1000 with overnight culture and shaken overnight at 30°C, to relieve selective pressure and to allow full integration of the cassette. After repeating this step, 10-fold dilutions of suspension were prepared and spread on LB agar/Km

plates for overnight growth at 30°C. Resulting single colonies were patched onto LB agar plates containing antibiotics to which the bacteria should be sensitive (Cm^S) and resistant (Km^R), and incubated overnight at 30°C. Chloramphenicol-sensitive (Cm^S) and Km^R clones indicate that the plasmid has been removed from the bacteria and that a successful cassette replacement has taken place. Cm^S and Km^R colonies were tested by PCR to confirm presence of the Km cassette in place of the target gene. Primer combinations which amplify the region where cassette meets chromosome were used: Next/Cext primers directed towards the cassette; and Km1/Km2 primers in the cassette facing outwards (Figure 7; Table 3).

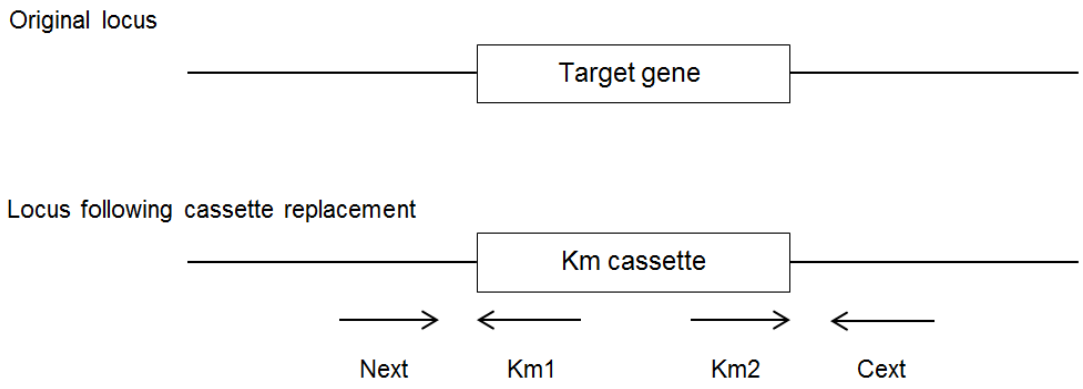

Figure 7. PCR verification of gene replacement. Primer combinations featuring inward facing Next/Cext primers amplifying cassette flanking regions, and outward facing Km1/Km2 within the cassette, were used to confirm the presence of the cassette.

4.13. Km cassette removal with pCP20

The temperature-sensitive plasmid pCP20 encodes Cm and ampicillin (Amp) resistance genes, and also possesses the FLP recombinase gene, *flippase* (*Flp*). pCP20 is used to remove the Km cassette from between FRT sites by site-specific recombination [218].

Km cassette-containing ECs were electroporated with 1 µg of plasmid pCP20. LB broth was added and cells shaken at 30°C. The suspension was centrifuged, SN discarded and the bacterial pellet re-suspended in LB broth. The sample was then incubated overnight on LB agar/Cm plates at 30°C for cassette excision. As

previously described, 30°C is sufficient for basal *flp* expression to mediate FRT recombination [218]. Resulting single colonies were re-streaked on LB agar/Cm plates and incubated overnight at 30°C. Single colonies were then patched on LB agar/Km and LB agar/Cm plates and incubated overnight at 30°C to screen for loss of the Km cassette and consequently loss of Km^R. Km^S and Cm^R clones were then streaked on LB agar plates and incubated overnight at 42°C to cure transformants of pCP20. Single colonies were patched on LB agar and LB agar/Cm plates and incubated overnight at 30°C to screen for loss of Cm^R, and therefore loss of pCP20. Cm^S clones were tested by PCR to confirm removal of the Km cassette [217].

4.14. Relative Survival Assay (RSA)

The RSA was performed as described previously [219], with minor modifications. Overnight culture was used to inoculate LB broth, which was grown to $O.D._{600}$ 0.6. One millilitre of $O.D._{600}$ 0.6 culture was centrifuged for 2 min at 12,000 rpm, and the SN discarded. The bacterial pellet was re-suspended in 1 ml 10 mM PBS (pH 6.5), centrifuged for 2 min at 12,000 rpm, and the SN discarded. This step was repeated three times, before final re-suspension in 1 ml 10 mM PBS (pH 6.5). A suspension was created of 10^5 CFU/ml in 10 mM PBS (pH 6.5), 1 % tryptone soy broth (TSB), and 100 mM NaCl. Five microlitre aliquots of this suspension was treated with different concentrations of PxB or colistin (0.033, 0.064, 0.096, 0.128, 0.192, 0.256 µg/ml), to a final volume of 30 µl made up with 10 mM PBS (pH 6.5). All samples were incubated statically for 1 hour at 37°C. Following incubation, samples were diluted serially in 10 mM PBS (pH 6.5) and 50 µl of the final dilution was plated on each half of an LB agar plate, in duplicate. After overnight incubation at 37°C, colonies were counted, CFU/ml determined, and the survival percentage of exposed bacteria calculated with respect to untreated samples.

4.15. Minimum Inhibition Concentration (MIC) assay

MICs were performed using the doubling agar dilution method as described previously [220, 221]. LB agar plates were made up with varying concentrations of antibiotics (Table 5). Overnight cultures were diluted to 10^{-4} in 1x PBS, then 400 µl of each culture added to a 37 well inoculation mould. The mould was placed onto a Denley Multipoint Inoculator, with the inoculation pins sterilised in 100% ethanol prior to use. Agar plates were inoculated, left briefly to dry and then incubated overnight at 37°C. In the morning, plates were scored and the MIC values determined where growth was no longer visible.

4.16. Data analysis

MS Excel was used to compile, format, and statistically analyse data.

Table 2. Strains used in this study

Strains	Comments
Ecl8	w/t *K. pneumoniae*
Ecl8Δ*ramA*	Ecl8: deleted of *ramA*
Ecl8Δ*ramR*	Ecl8: deleted of *ramR*
Ecl8<*ramA*>Km	Ecl8: *ramA* replaced with Km cassette
Ecl8<*romAramA*>Km	Ecl8: *romAramA* replaced with Km cassette
Ecl8<*ramR*>Km	Ecl8: *ramR* replaced with Km cassette
Ecl8<*ompC*>Km	Ecl8: *ompC* replaced with Km cassette
Ecl8Δ*ramR*<*ompC*>Km	Ecl8Δ*ramR*: *ompC* replaced with Km cassette
Ecl8<*ompF*>Km	Ecl8: *ompF* replaced with Km cassette
Ecl8Δ*ramR*<*ompF*>Km	Ecl8Δ*ramR*: *ompF* replaced with Km cassette
Ecl8<*acrAB*>Km	Ecl8: *acrAB* replaced with Km cassette
Ecl8Δ*ramR*<*acrAB*>Km	Ecl8Δ*ramR*: *acrAB* replaced with Km cassette
Ecl8<*tolC*>Km	Ecl8: *tolC* replaced with Km cassette
Ecl8Δ*ramR*<*tolC*>Km	Ecl8Δ*ramR*: *tolC* replaced with Km cassette
Ecl8<*lpxO*>Km	Ecl8: *lpxO* replaced with Km cassette
Ecl8Δ*ramR*<*lpxO*>km	Ecl8Δ*ramR*: *lpxO* replaced with Km cassette
4/74	w/t *S.*Typhimurium
4/74Δ*ramR*	4/74: deleted of *ramR*
4/74Δ*ramRA*	4/74: deleted of *ramRA*
4/74Δ*phoP*Δ*ramR*	4/74: deleted of *phoP* and *ramR*
4/74Δ*phoP*Δ*ramRA*	4/74: deleted of *phoP* and *ramRA*
4/74Δ*phoPQ*	4/74: deleted of *phoPQ*

Table 3. Primer list

Primer	Sequence	Source
Next *lpxO*	5' – TAT AGT TCC AGG GGA TAA CG – 3'	IDT
Cext *lpxO*	5' – GCC GAA GGG CTG GAC AAT CG – 3'	IDT
Next*phoPQ*	5' – CCG CGC TAG CCG CGG ATC G – 3'	IDT
Cext*phoPQ*	5' – GGG TGG CTA AGG CGT CCA G – 3'	IDT
Km1	5' – ATC CTG ATA TGA ATA AAT TGC – 3'	IDT
Km2	5' – ATC CAT GTT GGA ATT TAA TCG – 3'	IDT
*phoP*F_EcoRI	5' – CGG AAT TCA TAT AAA CCA TAC GGT AGT GA – 3'	IDT
*phoP*F_ScaI	5' – AAA AGT ACT TCA TCA GCG CAA TTC GAA – 3'	IDT
*lpxO*F_EcoRI	5' – CGG AAT TCC CGA CTT CAG CGA ATA GCG – 3'	IDT
*lpxO*R_ScaI	5' – AAA AGT ACT TTT TTA AAA CGC GCT CCA GAT – 3'	IDT
*lpxO*_qF	5' – CAG TGG TGA GAA TCG CTT GA – 3'	IDT
*lpxO*_qR	5' – ACC GCT TAC CAA CAA TAC GG – 3'	IDT

Table 4. Plasmid list

Plasmid	Source/Comment
pACYC184	Laboratory stock
pAC*lpxO*	This project
pAC*phoP*	This project
pACYC177	Laboratory stock
pAC*ramA*	Laboratory stock
pBR322	Laboratory stock
pBR*lpxO*	This project
pJET 1.2/blunt	Thermo Scientific CloneJet PCR Cloning Kit
pGEM-T Easy	Promega pGEM-T Easy Vector System
pTOF*lpxO*	Laboratory stock
pTOF*phoPQ*	Laboratory stock

Table 5. Antibiotic concentrations used

Antibiotic	Working concentrations (µg/ml)
Ampicillin (Amp)	100
Chloramphenicol (Cm)	50
Kanamycin (Km)	50
Tetracycline (Tc)	25
Colistin (Col)	RSA: 0.033, 0.064, 0.096, 0.128, 0.192, 0.256 MIC: 0.0156, 0.0234, 0.0312, 0.0468, 0.0625, 0.094, 0.125, 0.1875, 0.25, 0.375, 0.5, 1
Polymyxin B (PxB)	RSA: 0.033, 0.064, 0.096, 0.128, 0.192, 0.256 MIC: 0.0156, 0.0234, 0.0312, 0.0468, 0.0625, 0.094, 0.125, 0.1875, 0.25, 0.375, 0.5, 1
Tigecycline (Tig)	RSA: 0.033, 0.064, 0.096, 0.128, 0.192, 0.256 MIC: 0.0156, 0.0234, 0.0312, 0.0468, 0.0625, 0.094, 0.125, 0.1875, 0.25, 0.375, 0.5, 1, 2, 4

5. Results

5.1. Construction and validation of mutants in *K. pneumoniae*

The aims of this project were to characterise the contribution of the RamA transcription regulator and the PhoPQ two-component system in the regulation of *lpxO* in *K. pneumoniae*. RamA and PhoPQ are associated with mediating a resistance phenotype to cationic antimicrobials via their regulation of bacterial permeability and lipid A-modifying genes respectively [1, 177, 183, 196-199, 211]. Additionally, both have been linked to *lpxO* regulation in previous studies in *K. pneumoniae* [1, 183, 211]. We sought to determine the role of *lpxO* in generating a resistance phenotype to the last-line cationic polymyxins, PxB and colistin. Previous reports in *K. pneumoniae* have demonstrated that PhoPQ-regulated *lpxO* overexpression mediates CAMP resistance and immune evasion [183, 211], with a similar phenotype observed in strains overexpressing *ramA* [1].

To achieve these goals, we needed to generate *lpxO* and *phoPQ* knockout mutants in *K. pneumoniae* wild-type (w/t) Ecl8 and *ramA*-overexpressing Ecl8Δ*ramR* backgrounds. The removal of *lpxO* in these backgrounds would allow us to a) establish whether the presence of *lpxO* is necessary for PxB and colistin susceptibility; and b) determine if a functional *lpxO* gene in the presence of *ramA* overexpression is necessary to mediate polymyxin and CAMP susceptibility. The generation of Δ*phoPQ* mutants in w/t and *ramA*-overexpressing strains would allow us to confirm if RamA-mediated regulation of the *lpxO* gene is co-dependent on a functional *phoPQ* locus.

5.1.1. Validation of previously generated Δ*lpxO* mutants

The original plan for this project did not require construction of new Δ*lpxO* mutants due to the presence of existing Δ*lpxO* mutants in Ecl8 and Ecl8Δ*ramR* backgrounds; Ecl8<*lpxO*>Km and Ecl8Δ*ramR*<*lpxO*>Km respectively. These were generated previously using a gene exchange protocol, whereby the target *lpxO* gene is replaced with a kanamycin (Km) resistance cassette. Prior to performing characterisation experiments to assess the impact of gene loss on antimicrobial susceptibility, these mutants first had to be validated by PCR to confirm the loss of the *lpxO* gene. PCR screening using *lpxO* qPCR primers (Table 3) instead confirmed the presence of the gene on two separate occasions, indicating that the previously generated Ecl8<*lpxO*>Km and Ecl8Δ*ramR*<*lpxO*>Km mutants had in fact been unsuccessful (Figure 8). As a result, this required the construction of new

Δ*lpxO* mutants in order to begin characterising the role of *lpxO* in *K. pneumoniae* RamA-mediated AMR.

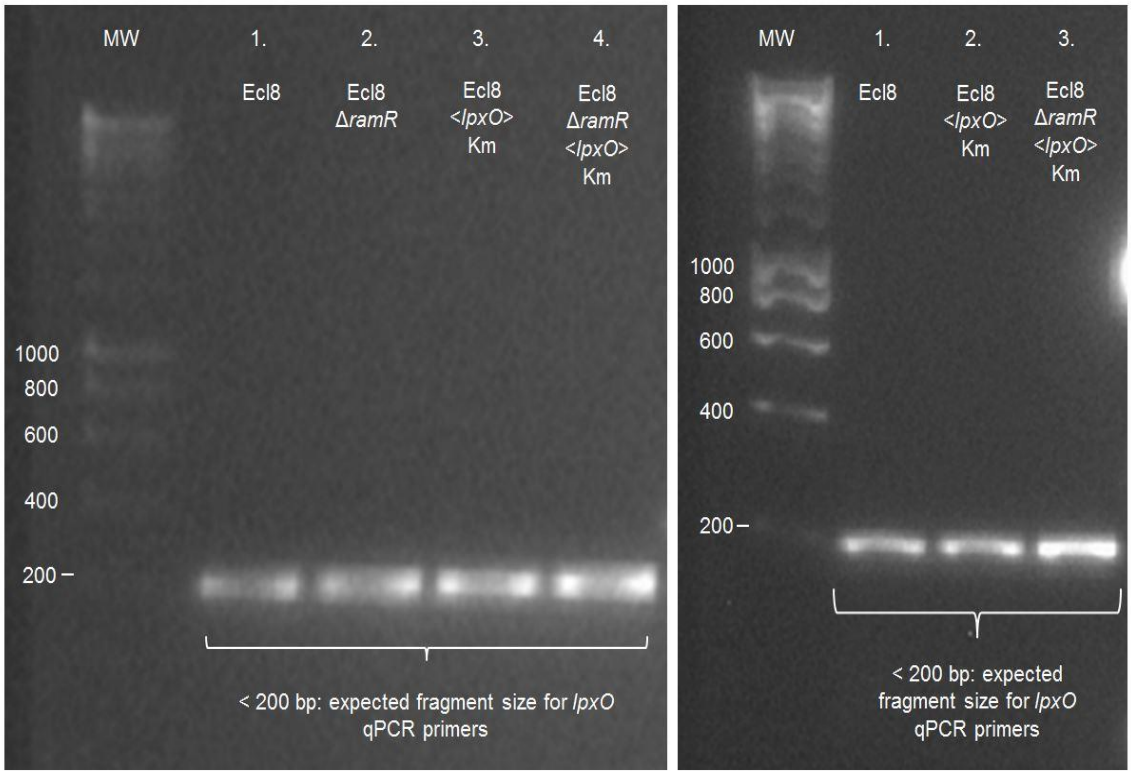

Figure 8. PCR validation of existing Δ*lpxO* mutants. On two separate occasions (shown in both panels), agarose gel electrophoresis of PCR products showed a 200 base pair (bp) band for all strains tested. This was equivalent to the expected fragment size for the *lpxO* qPCR primers used, indicating presence of the gene in all strains including putative mutants.

5.1.2. Genetic mutant construction methodology

In light of the confirmation that existing *lpxO* mutants were not as they appeared, we generated new *lpxO* and *phoPQ* knockout mutants. The exchange protocol required a pre-existing pTOF plasmid, for an accurate description of which refer to Merlin et al., 2002 [217]. The pTOF plasmid carries a gene-specific cassette containing Km and Cm resistance genes, with *lpxO*/*phoPQ* gene flanks (*lpxO*NoCo/*phoPQ*NoCo) cloned into it. The recombinant construct would then be introduced into either *K. pneumoniae* w/t Ecl8 or Ecl8Δ*ramR* by electroporation. Following a series of experimental steps involving incubations at 30°C or 42°C, the *lpxO* gene is replaced

by the Km cassette via a process of homologous recombination. The exchange protocol consisted of three key steps: integration of the cassette into the chromosome; purification of clones with the correct phenotype; and successful mutant generation, confirmed by a kanamycin resistant (Km^R) and chloramphenicol sensitive (Cm^S) phenotype and PCR screening for the presence of the cassette.

Electro-competent cells derived from Ecl8 and Ecl8Δ*ramR* were transformed with the temperature-sensitive pTOF plasmid containing the gene-specific (either *lpxO* or *phoPQ*), FRT-flanked Km cassette. Transformed cells were then incubated on LB agar/Km at 30°C overnight. The Km cassette allows growth and selection of plasmid-possessing bacteria in the presence of Km. Overnight incubation at 30°C keeps the temperature-sensitive plasmid viable, whilst homologous recombination takes place between the integrating Km cassette and the chromosomal region it specifies for. A second overnight incubation in LB broth/Km at 30°C was followed by growth overnight at 42°C on LB agar/Km. Km exposure maintains selection for the Km cassette, and the elevated temperature at 42°C prevents the plasmid from autonomously replicating, ensuring only cassette-chromosome integrants survive. Large single colonies, representing the desired recombinants, were picked and purified by re-streaking on LB agar/Km for overnight growth at 42°C. Single colonies were then pooled and incubated overnight at 30°C in LB broth to temporarily relieve selective pressure and allow recombination to complete. The final steps involved re-introduction of Km selection via serial dilution of overnight culture onto LB agar/Km and overnight incubation at 30°C. Single colonies were then patched onto LB agar plates containing either Cm or Km, with an expected Cm^S and Km^R phenotype for mutants with *lpxO* replaced by the Km cassette and no autonomously replicating plasmids. Having achieved the appropriate Km^R and Cm^S phenotype, PCR screens using primers specific for different possible Km cassette orientations were performed to confirm cassette integration and gene loss (Figure 7; Table 3).

The final stage of generating Δ*lpxO* and Δ*phoPQ* mutants required removal of the Km cassette with pCP20. The pCP20 plasmid possesses the *Flp* gene, encoding an FLP recombinase that removes the cassette from between the cassette's FRT flanking regions. Its removal would be the final step of mutant generation, ensuring that any phenotypes arising during antimicrobial susceptibility testing were a result of gene deletion, not an artefact of the Km cassette.

5.1.3. Challenges faced during mutant construction

Unfortunately, despite numerous attempts to generate these mutants, PCR screens for the presence of the cassette and the absence of either *lpxO* or *phoPQ* could not be confirmed, and the cassette removal stage was never reached. On several occasions, an unsuccessful exchange was confirmed due to the retention of Cm^R. However, from the majority of attempts, despite precisely following the temperature-specific steps which should select for correct integrants, and even though the Km^R and Cm^S phenotype was observed implying a successful exchange, genotypic tests could not confirm gene knockout. PCR screens to detect the Km cassette either failed to generate bands, or resulted in aspecific amplification that did not produce an expected PCR product size, indicating an unsuccessful exchange. In addition, follow-up PCRs with *lpxO* qPCR primers produced band sizes equivalent to <200 bp, comparable to the w/t positive control and indicative of gene presence. PCR screens were conducted multiple times using different machines to rule out equipment as the reason behind unsuccessful genotypic confirmation. Furthermore, phenotypic assessments were carried out for several of the putative mutants, for which PCR screens could not confirm gene loss. Antimicrobial susceptibility assays performed by agar dilution and disk diffusion methods on these strains showed values indistinguishable from w/t Ecl8.

The study from which the exchange protocol originates [217] reported a high protocol efficacy and success rate, whilst one of the paper's authors also confirmed no struggles generating mutants with the protocol (McAteer, S., Aug 2016, personal communication). Furthermore, the study's citation index demonstrates that the protocol is successfully reproducible [217]. Moreover, previous studies have demonstrated an ability to generate both *lpxO* and *phoPQ* inactivated mutants [183, 191, 222, 223], indicating that these genes are not essential for bacterial survival. As a result we continued to persevere with the protocol in an effort to generate mutants, but despite alterations to the protocol, such as maintaining antibiotic selection throughout, and direct training at the source laboratory where the method was developed, we did not achieve the desired outcome. As mutants had previously been successfully generated in *K. pneumoniae* Ecl8 using the same exchange protocol, including in our laboratory, we identified environmental conditions as a possible hurdle. We first confirmed that the incubators required for temperature-sensitive steps of the protocol were functioning correctly. We then began with fresh

reagents, including new LB broth and agar. However, none of these approaches succeeded in resolving the situation. Because the nature of the environmental conditions that might have been affecting the protocol's efficacy could not be pinpointed, we consequently attempted the exchange protocol in an external laboratory where mutants had been successfully generated in the past. Despite producing isolates with an appropriate Km^R phenotype, PCR screens again showed absence of the Km cassette and presence of *lpxO*.

5.2. Construction of complementation plasmids

As a result of our struggles to generate *lpxO* and *phoPQ* knockout mutants in Ecl8 and Ecl8Δ*ramR*, we set about with an adapted plan to examine the role of *lpxO* in the *K. pneumoniae* AMR phenotype. We had struggled to generate knockout mutants to analyse the effect of *lpxO* gene absence on resistance, therefore the new approach assessed if overexpression of the *lpxO* gene alone could affect susceptibility to the last-line drugs PxB, colistin, and tigecycline. These antimicrobial agents were selected due to the previously reported role of *ramA* overexpression in mediating resistance to tigecycline [134, 135, 143], and the impact of both *ramA* and *phoPQ* expression on reducing susceptibility to PxB and colistin [1, 177, 183, 196-199, 211]. Therefore, we sought to directly compare the effects of *ramA*, *phoP* and *lpxO* overexpression on susceptibility to these agents when introduced into the w/t Ecl8 and the *ramA* overexpressor Ecl8Δ*ramR*. We predicted that *lpxO* overexpression would reduce susceptibility to PxB and colistin, consistent with previous studies where lipid A modifications reduce the negative charge of the OM and limit the efficacy of CAMP binding and OM permeabilisation [176, 177, 183, 211]. While not fully representative of intrinsic naturally induced gene overexpression, our approach involved constructing *ramA*, *phoP* and *lpxO* overexpression plasmids, using vectors with established copy numbers, and assessing the direct effect of increased gene expression on bacterial susceptibility to PxB, colistin and tigecycline.

The construction of these overexpression plasmids also fulfilled a dual role as complementation plasmids. This was in the event that we successfully generated *lpxO* and *phoPQ* knockout mutants; we would need to confirm that any phenotypes observed as a result of gene loss could be recovered by the gene's re-introduction. Unfortunately, we were unable to utilise these plasmids for complementation due to the inability to generate knockout mutants.

5.2.1. Ecl8/pAC*lpxO* and Ecl8Δ*ramR*/pAC*lpxO*: *lpxO* overexpression in *K. pneumoniae*

Plasmid copy number refers to the average number of plasmid copies in the bacterial cell, and is dependent on plasmid size and the size of the inserted gene, as well as the plasmid's origin of replication. For the purposes of our experiments, we aimed to increase *lpxO* levels via overexpression using a low copy plasmid. We did

not want to overwhelm the bacteria carrying the construct with an unnaturally high level of *lpxO* as doing so could potentially result in erroneous phenotypes consistent with the stress response e.g. structural issues within the bacterium but not actual functional phenotypes. Based on our requirements, we chose pACYC184 for our destination vector, which would carry the *lpxO* insert and be used in later antimicrobial susceptibility assays. pACYC184 is a pACYC plasmid; these plasmids are classed as low copy number with between ten to twelve copies per cell [224].

To generate our plasmid constructs, we first created the *lpxO* insert by amplifying the *lpxO* gene from Ecl8 genomic DNA using primers with EcoRI and ScaI restriction sites specific to our final pACYC184 destination vector (Table 3). The *lpxO* insert was next ligated into the pJET cloning vector and transformed into *E. coli* DH10β cells via heat-shock transformation. Resulting transformants were picked and screened by colony PCR with *lpxO*- or pJET vector-specific primers to determine whether a successful ligation between insert and vector had taken place. Following this, the pJET*lpxO* plasmid was extracted and restriction digests performed using the primer-specific EcoRI and ScaI restriction sites to release the fragment. Following gel extraction, the *lpxO* fragment was successfully ligated into pACYC184, transformed into *E. coli* DH10β, and the subsequent transformants then screened. pACYC184 possesses both tetracycline (Tc) and Cm resistance genes. The excision of pACYC184 with EcoRI and ScaI and subsequent ligation of the *lpxO* insert disrupts the Cm resistance gene; therefore the transformants were screened using the phenotypic loss of Cm^R and PCR presence of the *lpxO* insert.

To assess the effect of *lpxO* overexpression in *K. pneumoniae*, pAC*lpxO* was extracted from DH10β/pAC*lpxO*, with a small amount taken aside and digested with EcoRI and ScaI to confirm the presence of the *lpxO* insert. The remaining plasmid was introduced into Ecl8 and Ecl8Δ*ramR* by electroporation and selected for using Tc. In order to determine whether any observed effects from subsequent antimicrobial susceptibility experiments were a result of increased *lpxO* expression or from the influence of the vector, we set up plasmid-only controls. pACYC184 from laboratory stocks was extracted and transformed into Ecl8 and Ecl8Δ*ramR* by electroporation. Tc^R transformants were picked and stored for later use as controls.

5.2.2. Ecl8/pBR*lpxO* and Ecl8Δ*ramR*/pBR*lpxO*: further increasing *lpxO* levels

In addition to pAC*lpxO*, we wanted to generate an alternative *lpxO* overexpression construct with a higher copy number, in case the *lpxO* increase mediated by pAC*lpxO* failed to influence an AMR phenotype. We therefore selected pBR322, a medium copy number plasmid generating between fifteen to twenty copies per cell [224]. pBR322 from laboratory stocks was extracted and digested using the primer-specific EcoRI and ScaI restriction enzymes, while pAC*lpxO* was simultaneously cut with the same enzymes. Following gel extraction the *lpxO* fragment was ligated into pBR322 and transformed into *E. coli* DH10β, with transformants subsequently screened. pBR322 possesses both ampicillin (Amp) and Tc resistance genes. The excision of pBR322 with EcoRI and ScaI and subsequent ligation of the *lpxO* insert disrupts the Amp resistance gene; therefore the transformants were screened for a Tc^R and Amp^S phenotype and PCR presence of *lpxO*. The pBR*lpxO* construct was then extracted from DH10β/pBR*lpxO* and electroporated into Ecl8 and Ecl8Δ*ramR*; a Tc^R and Amp^S phenotype confirmed the successful transformation. Plasmid-only controls were also set up, where pBR322 was electroporated into Ecl8 and Ecl8Δ*ramR*, with successful clones selected for with Tc.

5.2.3. Ecl8/pAC*phoP* and Ecl8Δ*ramR*/pAC*phoP*: *phoP* overexpression in *K. pneumoniae*

In order to assess the effect of *phoP* overexpression on reduced antimicrobial susceptibility, we first had to generate the plasmid construct similarly to pAC*lpxO*. However, pAC*phoP* proved considerably more difficult to generate. Originally, the *phoP* insert was amplified from Ecl8 genomic DNA, using primers with EcoRI and ScaI restriction sites specific to pACYC184 (Table 3), and ligated into pJET. Despite the relative ease with which the *lpxO* insert had ligated into this vector, upon transformation of pJET*phoP* into *E. coli* DH10β, transformants consistently failed to grow. As a result, we resorted to changing our vector to the pGEM-T Easy cloning system. This required an intermediary step prior to ligation to A-tail our *phoP* insert. This was because the initial *phoP* insert was amplified with Q5 proof-reading polymerase which generates blunt ends, whereas the pGEM-T Easy vector requires an A-tailed insert for ligation. Following transformation into DH10β, colony PCR screening of resulting transformants with amplification primers confirmed the presence of *phoP*. Subsequent digests of extracted pGEM-T Easy*phoP* and the destination vector pACYC184 with primer-specific EcoRI and ScaI were followed by

ligation and transformation into DH10β. Transformants were selected for using Tc, with a Tc^R phenotype confirming a successful ligation, and the plasmid was then extracted and electroporated into Ecl8 and Ecl8Δ*ramR*. No control strains had to be generated for pAC*phoP* due to the Ecl8/pACYC184 and Ecl8Δ*ramR*/pACYC184 strains already established alongside pAC*lpxO* generation.

5.2.4. Ecl8/pAC*ramA* and Ecl8Δ*ramR*/pAC*ramA*: *ramA* overexpression in *K. pneumoniae*

In order to assess the effect of *ramA* on antimicrobial susceptibility, we needed to compare the *lpxO* and *phoPQ* overexpression constructs with a similarly generated *ramA*-overexpressing construct. Fortunately, pAC*ramA* had been previously constructed, therefore it was extracted from lab stocks and electroporated into Ecl8 and Ecl8Δ*ramR.* Controls were established in order to determine any influence from the pAC*ramA* vector on susceptibility results; pACYC177 from lab stocks was extracted and electroporated into Ecl8 and Ecl8Δ*ramR*. Ecl8 and Ecl8Δ*ramR* transformants with pAC*ramA* and pACYC177 were selected for using Km; a Km^R phenotype confirmed the successfully transformed constructs.

5.3. Resistance phenotypes of different mutants and overexpression constructs

In order to define the contribution of *lpxO* to *K. pneumoniae* RamA-mediated AMR, and to determine any potential role for *phoPQ* in this phenotype, we performed antimicrobial susceptibility experiments. These consisted of MICs and relative survival assays (RSAs), which allowed the direct comparison of the susceptibility of strains overexpressing *lpxO*, *ramA*, or *phoPQ*, or with various RamA-regulated resistance genes knocked out. The subsequent relative susceptibility of these strains to PxB and colistin would provide indications as to the role of specific genes in generating resistance to polymyxin antibiotics. In addition, we were keen to assess the susceptibility of our strains to tigecycline for two reasons. Firstly, due to the established role of RamA and AcrAB in conferring resistance to tigecycline [126, 225-230], we aimed to use tigeycline as a control; to validate our strains and plasmid constructs by comparing reported phenotypes with our own outcomes. Secondly, as described previously, tigecycline is a drug of increasing interest in the treatment of MDR *K. pneumoniae* as it retains good effectiveness against β-lactamase producing strains [144-146]. Therefore, examining mechanisms that contribute to tigecycline resistance is important in order to determine how to make this agent more effective.

In order to characterise the roles of our overexpressed genes, we needed to perform antimicrobial susceptibility experiments with a variety of *K. pneumoniae* strains. These were either the w/t Ecl8, strains possessing our gene-overexpression plasmids, or knockout mutants with a Km cassette replacing genes identified to be important in the RamA-mediated AMR phenotype. We performed both MIC experiments and RSAs in order to rigorously examine antimicrobial susceptibility phenotypes. RSAs are useful to investigate antimicrobial susceptibility as they are understood to provide an advantage to the drug in killing bacteria. Therefore RSAs allowed us to characterise and assess the ability of genes to promote bacterial survival under bactericidal challenge. MICs on the other hand provide an advantage to the bacteria, as the aim of these experiments is to assess bacterial growth inhibition, as opposed to bacterial survival. For these reasons we performed both MICs and RSAs to thoroughly characterise *lpxO*, *ramA* and *phoP* overexpression from a position of advantage to both the bug and the drug, and therefore provide extra support to any susceptibility trends observed.

MICs to tigecycline, PxB and colistin were tested by the doubling agar dilution method as described previously [220, 221]. Diluted bacterial cultures were inoculated on antibiotic-containing LB agar, grown overnight at 37°C, and assessed the next day for the concentration at which growth was inhibited. The concentrations of tigecycline tested ranged from 0.0156 mg/L to 4 mg/L; the concentrations tested for PxB and colistin ranged from 0.0156 mg/L to 1 mg/L. Between 0.0156 mg/L and 0.5 mg/L, each concentration increased by approximately 75%; between 0.5 mg/L and 4 mg/L, concentrations doubled (Table 5). Strains were tested in duplicate, and in some cases exhibited more than one MIC value. Therefore in-text references to MIC values use the highest concentration that was observed.

RSAs were conducted to assess the ability of *lpxO*, *ramA* and *phoP* overexpression to promote bacterial survival following exposure to increasing concentrations of PxB and colistin. RSAs were performed as described previously [219]: briefly, overnight cultures were incubated with or without antibiotics for one hour at 37°C, then serially diluted and spread on LB agar plates. After overnight incubation at 37°C, single colonies were counted and the CFU/ml calculated to 10^5. The relative survival was calculated as a percentage of the CFU/ml of surviving exposed bacteria at each concentration compared to corresponding unexposed bacteria. This assay provides an analysis of how specific genes contribute to survival in the face of intensifying antimicrobial pressure.

5.3.1. Characterising the contribution of *ramA* and RamA-regulated permeability genes to tigecycline, PxB and colistin susceptibility

Overexpression of *ramA* has been reported to mediate a MDR phenotype via its regulation of bacterial permeability genes [1, 125, 140], resulting in reduced susceptibility to tigecycline, PxB and colistin [1, 134, 135, 143]. To confirm these previous findings [1, 134, 135, 143], we tested w/t Ecl8, *ramA*-overexpressing Ecl8Δ*ramR*, and the *ramA*-deleted Ecl8Δ*ramA*. These also acted as controls to compare other tested strains to. We also included Ecl8<*ramA*>Km, Ecl8<*romAramA*>Km, and Ecl8<*ramR*>Km, generated previously in the lab using the Km cassette exchange protocol. Both sets of marked and unmarked deletion strains were tested to determine if there were any differences in susceptibility; to assess if the presence of the Km cassette has any influence on the bacterial susceptibility phenotype. Because we wanted to test the effect of plasmid-mediated *lpxO* and *phoP* overexpression, we also included Ecl8/pAC*ramA* and

Ecl8Δ*ramR*/pAC*ramA*, as well as the Ecl8/pACYC177 and Ecl8Δ*ramR*/pACYC177 vector-only controls. This allowed us to directly compare the influence of the three genes of interest – *lpxO*, *phoP*, and *ramA* – using the same plasmid-mediated expression mechanism.

In addition we tested knockout mutants of several RamA-regulated, resistance-associated permeability genes in the AMR phenotype, which had previously been generated using the Km cassette exchange protocol. The AcrAB-TolC efflux pump has been strongly linked with AMR due to alterations in efflux activity [122, 148] and is regulated at gene level by RamA [1]. We tested the susceptibility of Ecl8<*acrAB*>Km, Ecl8Δ*ramR*<*acrAB*>Km, Ecl8<*tolC*>Km, and Ecl8Δ*ramR*<*tolC*>Km in order to define which of the *acrAB* and *tolC* efflux genes might be most crucial to promoting reduced drug susceptibility. We also tested knockout mutants of the porin genes *ompC* and *ompF* – Ecl8<*ompC*>Km, Ecl8Δ*ramR*<*ompC*>Km, Ecl8<*ompF*>Km, and Ecl8Δ*ramR*<*ompF*>Km – due to their regulation by RamA [1, 140, 231] and their role in MDR, where their decreased expression leads to reduced influx [105, 152, 232, 233].

5.3.1.1. Overexpression of *ramA* the key factor in reducing susceptibility to tigecycline

MIC experiments exposing various *K. pneumoniae* strains to tigecycline demonstrate that *ramA* overexpression dramatically reduces susceptibility (Figure 9; Table 6). In Ecl8 and Ecl8Δ*ramA* (MIC = 0.25 mg/L and 0.1875 mg/L respectively), the MICs were approximately ten-fold lower than Ecl8Δ*ramR* (MIC = 2 mg/L). These MIC values were reinforced by similar differences between Ecl8<*ramR*>Km (MIC = 2 mg/L), and Ecl8<*ramA*>Km and Ecl8<*romAramA*>Km (MIC = 0.1875 mg/L for both). Indeed, with a few exceptions, strains with an Ecl8Δ*ramR* background where *ramA* is chromosomally overexpressed display a ten-fold higher MIC value compared to Ecl8, Ecl8Δ*ramA*, Ecl8<*ramA*>Km and Ecl8<*romAramA*>Km. Corresponding to the report by Majumdar et al. [1], we show that *ramA* inactivation does not appear to substantially reduce tigecycline susceptibility compared to w/t Ecl8. Additionally, retention of the Km cassette appeared to have no significant effect on the susceptibility phenotype, as seen by the similar MICs of the different *ramR* knockouts, and the various *ramA* knockout strains.

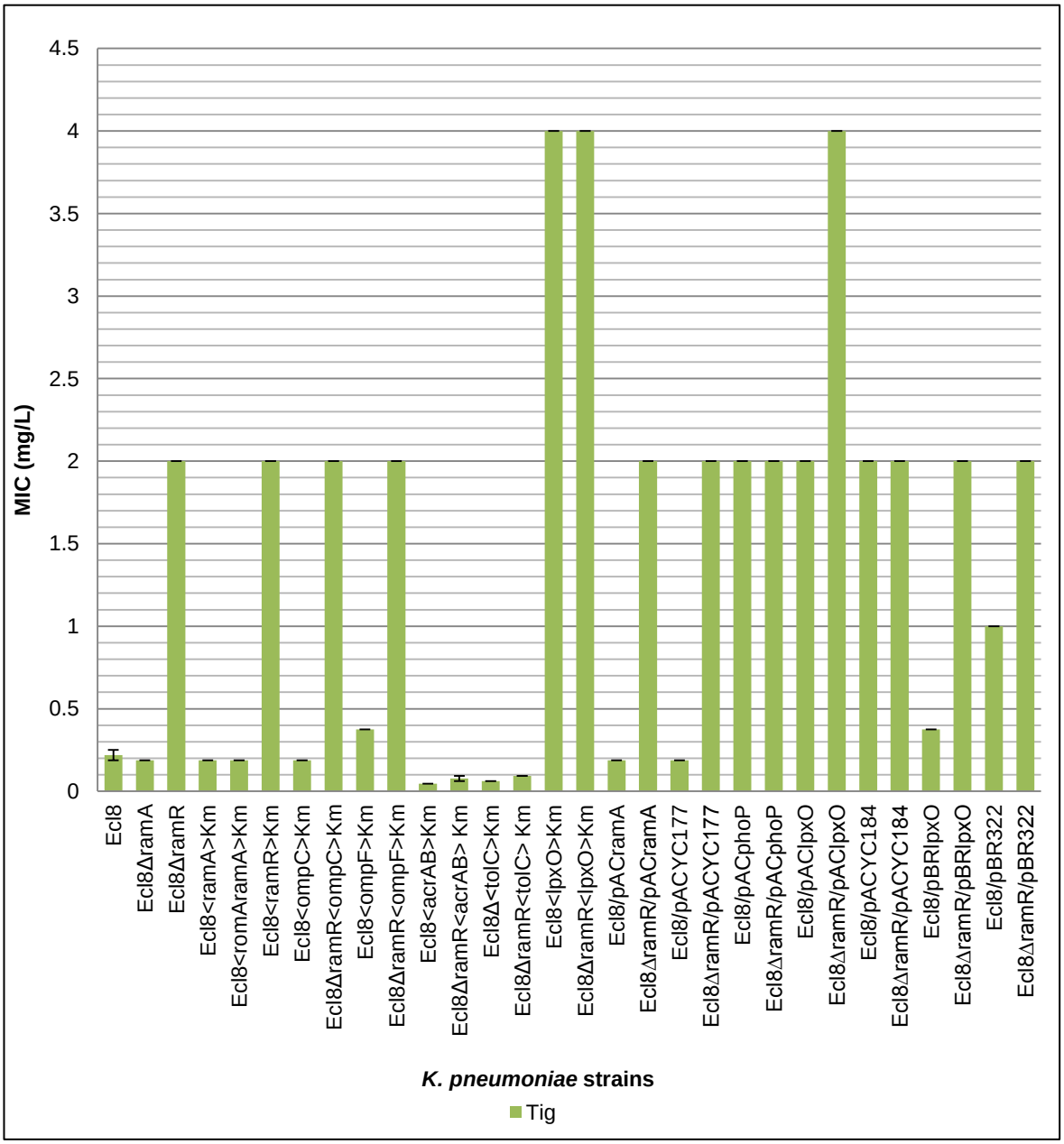

Figure 9. MIC of *K. pneumoniae* Ecl8 w/t and mutant strains to tigecycline. MICs are presented as an average of the experimentally derived results. Each strain was tested at least once, in duplicate. Error bars show the standard deviation of samples' MIC values.

Notably, Ecl8/pAC*ramA* (MIC = 0.1875 mg/L) and Ecl8Δ*ramR*/pAC*ramA* (2 mg/L) possessed the same MIC values as Ecl8 and Ecl8Δ*ramR* respectively (Figure 9; Table 6). In addition, the MICs of Ecl8/pAC*ramA* and Ecl8Δ*ramR*/pAC*ramA* were identical to the corresponding pACYC177 controls. Due to the established role of

ramA overexpression in conferring tigecycline resistance [134, 135, 143], and based on our own observations of this contribution, it seemed unusual that plasmid-mediated *ramA* overexpression was unable to further reduce susceptibility. This might indicate a problem with the generation of our pAC*ramA* strains, or could be because the pAC*ramA* plasmid incurs a fitness cost when introduced into the *K. pneumoniae* strain [234].

The RamA-regulated AcrAB-TolC efflux pump is an important component of RamA-mediated resistance to tigecycline [227, 228, 235-237]. Here we show that *tolC* and *acrAB* knockout mutants in the w/t Ecl8 background exhibit a minor increase in susceptibility compared to Ecl8 (Figure 9; Table 6). Notably, two exceptions to the observation that strains with an Ecl8Δ*ramR* background are more resistant than w/t Ecl8 are Ecl8Δ*ramR*<*tolC*>Km and Ecl8Δ*ramR*<*acrAB*>Km. Compared to the high MIC of Ecl8Δ*ramR* (MIC = 2 mg/L), Ecl8Δ*ramR*<*tolC*>Km and Ecl8Δ*ramR*<*acrAB*>Km are significantly more susceptible, with MIC values 20-fold lower (MIC = 0.094 mg/L for both) (Figure 9; Table 6). This indicates that the *acrAB* and *tolC* genes are important components of the RamA-regulated tigecycline resistance phenotype, consistent with previous findings [227, 228, 235-237], but does not elucidate which, if any, of these two genes is most vital to reducing tigecycline susceptibility. No differences were seen for *ompC* or *ompF* knockout mutants compared to the corresponding Ecl8 or Ecl8Δ*ramR* controls, except Ecl8<*ompF*>Km which demonstrated reduced susceptibility compared to w/t Ecl8 (MIC = 0.375 mg/L and 0.25 mg/L respectively). This corresponds to previously reported AMR phenotypes resulting from decreased *ompF* expression, which leads to reduced porin-mediated antimicrobial influx [152].

Table 6. MIC of *K. pneumoniae* Ecl8 w/t and mutant strains to PxB, colistin, and tigecycline

Strains	PxB (mg/L)	Colistin (mg/L)	Tigecycline (mg/L)
Ecl8	0.1875[2]	0.094 – 0.1875[2]	0.1875 – 0.25[2]
Ecl8Δ*ramA*	0.125 – 0.1875[2]	0.094 – 0.1875[2]	0.1875[2]
Ecl8Δ*ramR*	0.25[2]	0.1875[2]	2[2]
Ecl8<*ramA*>Km	0.1875[1]	0.125[1]	0.1875[1]
Ecl8<*romAramA*>Km	0.125 – 0.1875[1]	0.125[1]	0.1875[1]
Ecl8<*ramR*>Km	0.25[1]	0.1875[1]	2[1]
Ecl8<*ompC*>Km	0.1875[1]	0.125[1]	0.1875[1]
Ecl8Δ*ramR*<*ompC*>Km	0.25[1]	0.1875[1]	2[1]
Ecl8<*ompF*>Km	0.1875[1]	0.125[1]	0.375[1]
Ecl8Δ*ramR*<*ompF*>Km	0.25[2]	0.1875[1]	2[1]
Ecl8<*acrAB*>Km	0.1875 – 0.25[2]	0.1875 – 0.25[2]	0.0468[1]
Ecl8Δ*ramR*<*acrAB*> Km	0.1875[2]	0.125[2]	0.0625 – 0.094[1]
Ecl8Δ<*tolC*>Km	0.125 – 0.1875[2]	0.125[2]	0.0625[1]
Ecl8Δ*ramR*<*tolC*> Km	0.1875 – 0.25[2]	0.1875[2]	0.094[1]
Ecl8<*lpxO*>Km	0.375[2]	0.5 - 1[2]	4[1]
Ecl8Δ*ramR*<*lpxO*>Km	0.375[2]	0.5 - 1[2]	4[2]
Ecl8/pAC*ramA*	0.1875[2]	0.125 – 0.1875[2]	0.1875[1]
Ecl8Δ*ramR*/pACramA	0.25[1]	0.1875[1]	2[1]

Ecl8/pACYC177	0.125 – 0.1875[2]	0.125 – 0.1875[2]	0.1875[1]
Ecl8Δ*ramR*/pACYC177	0.1875 – 0.25[1]	0.125[1]	2[1]
Ecl8/pAC*phoP*	0.5 - 1[1]	0.375 – 0.5[1]	2[1]
Ecl8Δ*ramR*/pACphoP	1[1]	1[1]	2[1]
Ecl8/pAC*lpxO*	0.1875[2]	0.125 – 0.1875[2]	2[1]
Ecl8Δ*ramR*/pAClpxO	0.1875 – 0.25[1]	0.125 – 0.1875[1]	4[1]
Ecl8/pACYC184	0.125 – 0.1875[2]	0.094 – 0.1875[2]	2[1]
Ecl8Δ*ramR*/pACYC184	0.1875[1]	0.125[1]	2[1]
Ecl8/pBR*lpxO*	0.125[1]	0.094[1]	0.375[1]
Ecl8ΔramR/pBRlpxO	0.1875[1]	0.125 – 0.1875[1]	2[1]
Ecl8/pBR322	0.25[1]	0.1875[1]	1[1]
Ecl8ΔramR/pBR322	0.1875[1]	0.125[1]	2[1]

Table 6. Each strain was tested at least once, in duplicate. For in-text referencing of MIC values, the higher MIC is used. [1] Strains were tested once, in duplicate; [2] strains were tested twice, each time in duplicate; [3] strains were tested on three occasions, each time in duplicate.

5.3.1.2. Overexpression of *ramA* confers minimal resistance to PxB and colistin

In *K. pneumoniae*, *ramA* overexpression has previously been reported to mediate a reduction in susceptibility to both PxB and Colistin [1]. From our MIC experiments (Figure 10; Table 6), we show that in response to PxB exposure, *ramA*-overexpressing Ecl8Δ*ramR* and Ecl8<*ramR*>Km have a 1.3-fold higher MIC (MIC = 0.25 mg/L for both) than Ecl8, Ecl8Δ*ramA*, Ecl8<*ramA*>Km and

Ecl8<*romAramA*>Km (MIC = 0.1875 mg/L for all). These results show that *ramA* overexpression conferred a slight reduction in susceptibility to PxB; however *ramA* inactivation did not increase susceptibility. MICs after colistin exposure gave mixed results (Figure 10; Table 6). Ecl8<*ramR*>Km (MIC = 0.1875 mg/L) had a 1.5-fold higher MIC than Ecl8<*ramA*>Km and Ecl8<*romAramA*>Km (MIC = 0.125 mg/L for both), but presented the same MIC value as Ecl8, Ecl8Δ*ramA* and Ecl8Δ*ramR* (MIC = 0.1875 mg/L for all).

Similarly to tigecycline MICs, the introduction of pAC*ramA* into Ecl8 and Ecl8Δ*ramR* made no difference to PxB susceptibility, with these strains possessing the same MIC values as the corresponding Ecl8/pACYC177 and Ecl8Δ*ramR*/pACYC177 vector-only controls (MIC = 0.1875 mg/L and 0.25 mg/L respectively) (Figure 10; Table 6). pAC*ramA* also had no effect in reducing susceptibility to colistin (Figure 10; Table 6); interestingly, introduction of pACYC177 into Ecl8Δ*ramR* increased susceptibility. Because of the reported role of *ramA* overexpression in conferring tigecycline, PxB, and colistin resistance [1, 134, 135, 143], it is surprising that introduction of pAC*ramA* into our strains was unable to reduce susceptibility to any of these drugs.

Indeed, from the RSA results (Figures 11 and 12), Ecl8/pAC*ramA* and Ecl8Δ*ramR*/pAC*ramA* did not show any ability to reduce susceptibility to PxB or colistin. In fact, the only strains showing a statistically significant relative survival percentage after PxB exposure were Ecl8Δ*ramR* at 0.033 µg/ml (8.27 %), and Ecl8Δ*ramR*/pACYC177 at 0.033 µg/ml and 0.064 µg/ml (7.26 % and 1.41 % respectively). Following colistin treatment, the only statistically significant survival was by Ecl8Δ*ramR* at 0.033 µg/ml (9.8 %), Ecl8Δ*ramR*/pACYC177 at 0.033 µg/ml and 0.096 µg/ml (3.63% and 0.07 % respectively), and Ecl8Δ*ramR*/pAC*ramA* at 0.064 µg/ml (0.73 %). It is unusual that the pAC*ramA* construct was unable to confer resistance to PxB, and while Ecl8Δ*ramR*/pAC*ramA* did demonstrate survival after colistin treatment, it is more likely that this effect was a result of the Ecl8Δ*ramR* background. The proposed ineffectiveness of pAC*ramA* is supported by the MIC values of the pAC*ramA* strains for all the antibiotics tested. It is further reinforced by the RSA results, where the Ecl8Δ*ramR*/pACYC177 control possesses a similar ability to survive PxB and colistin exposure as Ecl8Δ*ramR*/pAC*ramA*. It is therefore likely that the survival demonstrated by strains in the RSAs is a result of the chromosomal *ramA* expression present in Ecl8Δ*ramR*. Indeed, all of the strains that

displayed statistically significant survival after PxB or colistin exposure had an Ecl8Δ*ramR* background in common, thus supporting previous data that *ramA* overexpression does confer protection against PxB and colistin killing. Furthermore, in subsequent RSAs testing pAC*lpxO* and pAC*phoP* strains (Figures 13 and 14) Ecl8Δ*ramR* again demonstrated statistically significant survival.

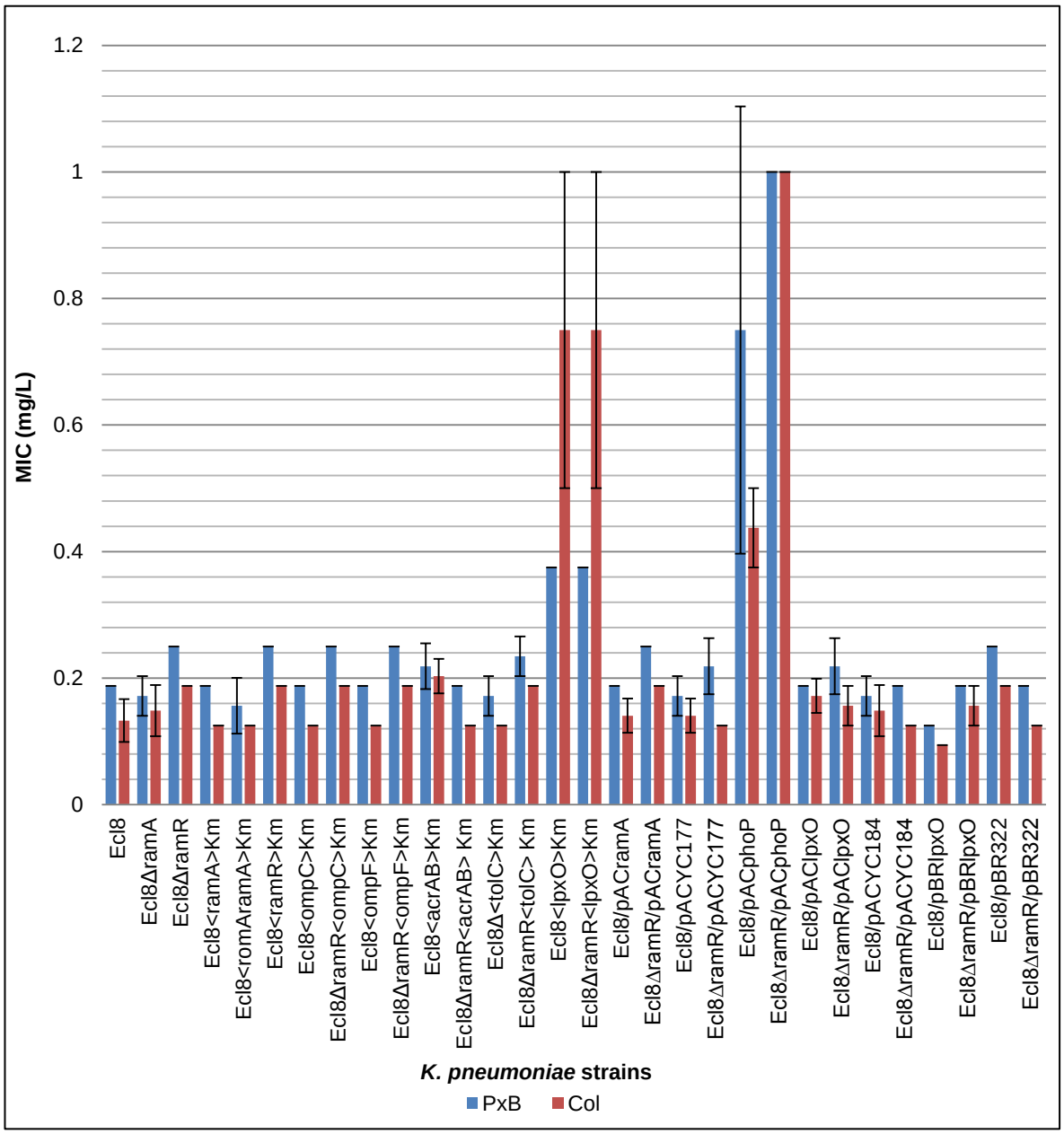

Figure 10. MICs of *K. pneumoniae* Ecl8 w/t and mutant strains to PxB and Colistin. MICs are presented as an average of the experimentally derived results. Each strain was tested at least once, in duplicate. Error bars show the standard deviation of samples' MIC values.

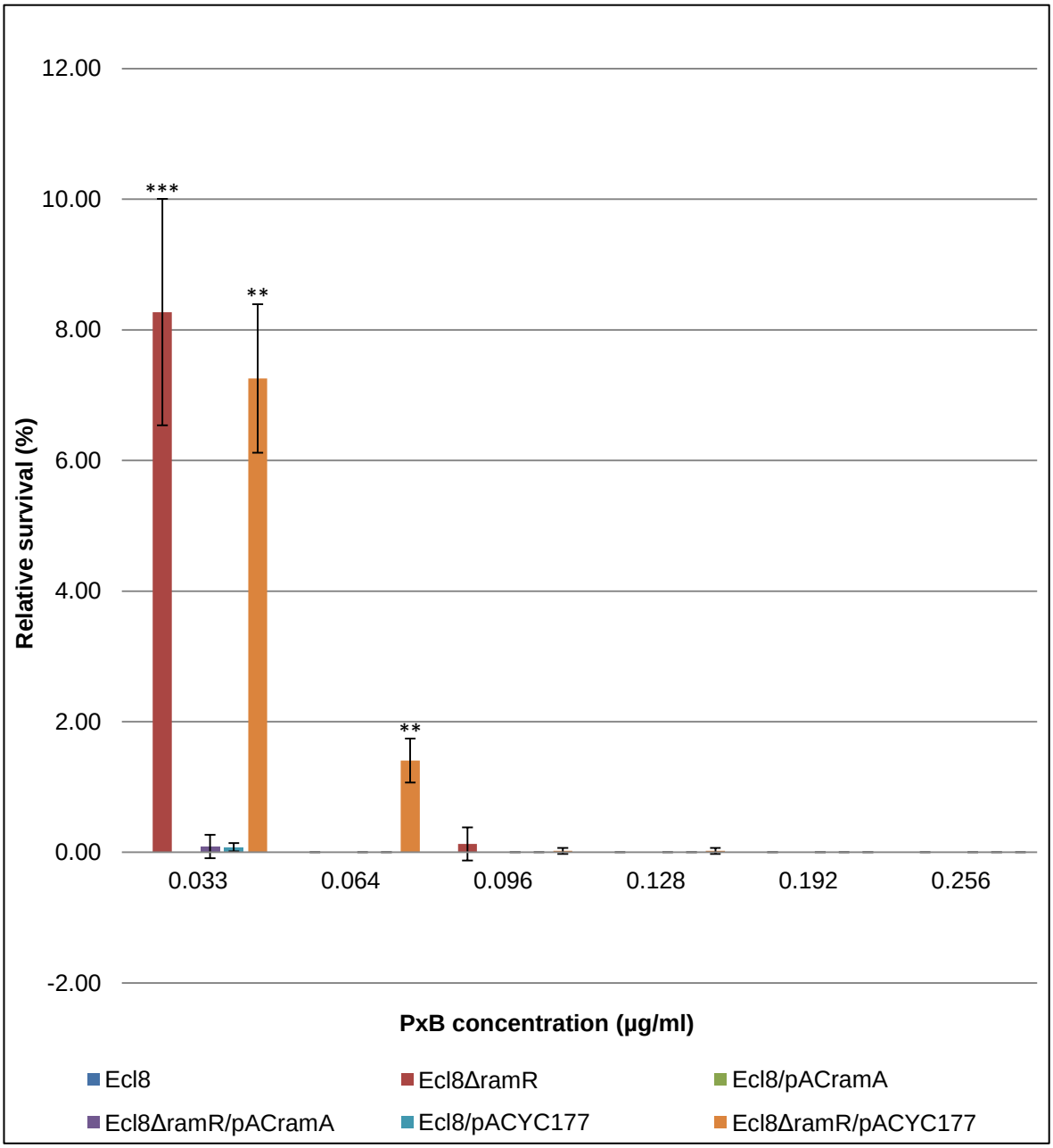

Figure 11. Relative survival assay of *K. pneumoniae* (Ecl8 w/t, mutant, pAC*ramA*, pACYC177 strains) to PxB. Bacteria were incubated stationary, with or without PxB, for 1 hour at 37°C before being serially diluted and 50 µl spread on LB agar half-plates in duplicate. After overnight incubation at 37°C, single colonies were counted and CFU/ml calculated to 10^5. The relative survival was calculated as a percentage of surviving exposed bacteria compared to corresponding unexposed bacteria. Error bars show the standard deviation of the relative survival percentages derived from the replicates. *Student's T-test* was performed on samples at each antibiotic concentration to determine if the CFU/ml was statistically significant compared to the w/t Ecl8 control at the same concentration: * for p <0.05, ** for p <0.005, *** for p <0.0005. Results show data from one experiment, performed in quadruplicate.

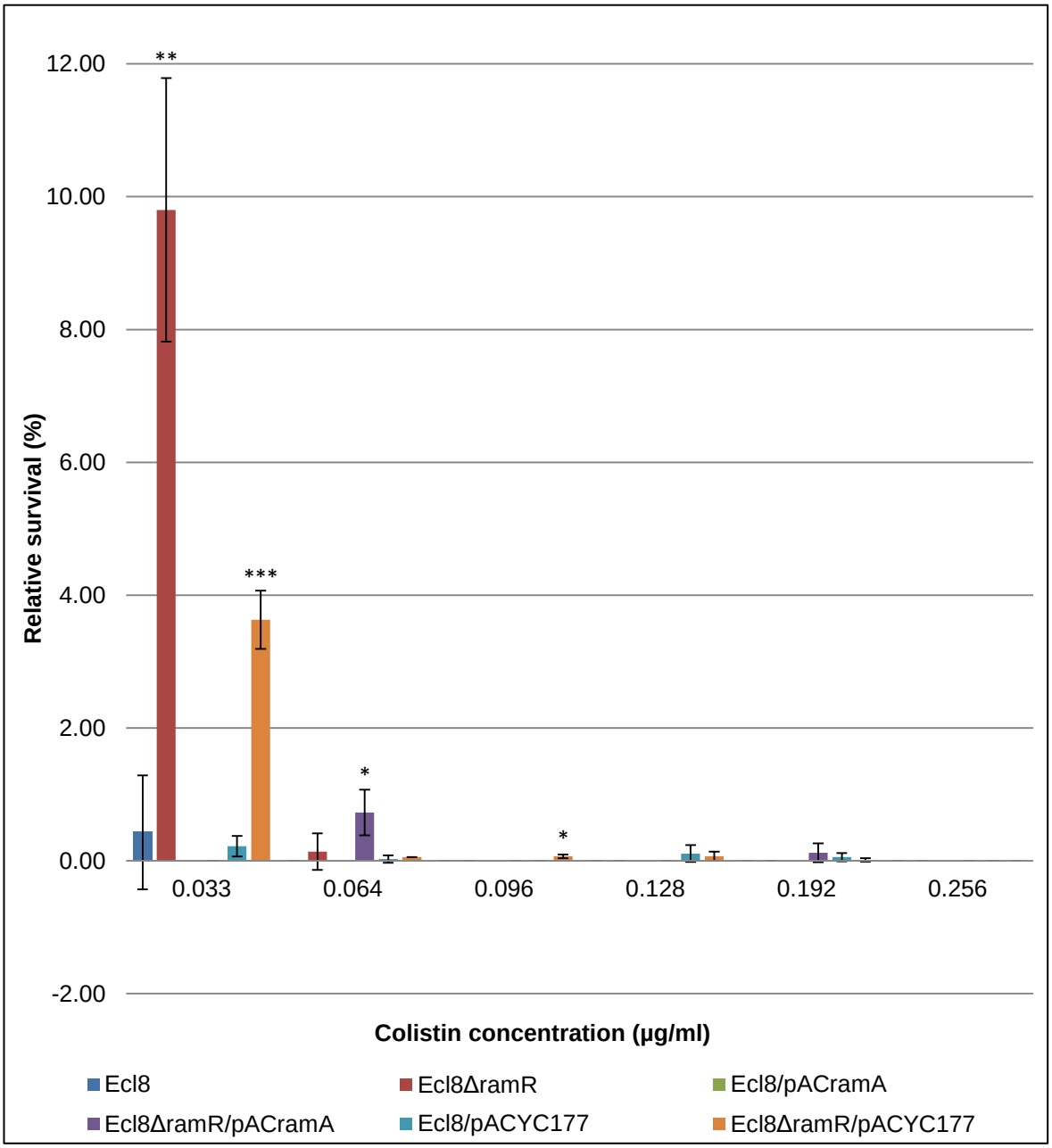

Figure 12. Relative survival assay of *K. pneumoniae* (Ecl8 w/t, mutant, pAC*ramA*, pACYC177 strains) to colistin. Bacteria were incubated stationary, with or without colistin, for 1 hour at 37°C before being serially diluted and 50 µl spread on LB agar half-plates in duplicate. After overnight incubation at 37°C, single colonies were counted and CFU/ml calculated to 10^5. The relative survival was calculated as a percentage of surviving exposed bacteria compared to corresponding unexposed bacteria. Error bars show the standard deviation of the relative survival percentages derived from the replicates. *Student's T-test* was performed on samples at each antibiotic concentration to determine if the CFU/ml was statistically significant compared to the w/t Ecl8 control at the same concentration: * for p <0.05, ** for p <0.005, *** for p <0.0005. Results show data from one experiment, performed in quadruplicate.

The Δ*acrAB* and Δ*tolC* mutants produced mixed responses to PxB and colistin (Figure 10; Table 6). Of the Δ*acrAB* mutants, Ecl8<*acrAB*>Km (PxB and colistin MIC = 0.25 mg/L) possessed a 1.3-fold higher PxB and colistin MIC compared to Ecl8 (PxB and colistin MIC = 0.1875 mg/L), whilst on the other hand Ecl8Δ*ramR*<*acrAB*>Km (PxB MIC = 0.1875 mg/L; colistin MIC = 0.125 mg/L) was approximately 1.3-fold more susceptible to both antimicrobials compared to Ecl8Δ*ramR* and Ecl8<*ramR*>Km (PxB MIC = 0.25 mg/L for both; colistin MIC = 0.1875 mg/L for both). Overexpression of *ramA* increases expression of *acrA* [1], and *acrAB* has been established as a critical component of RamA-mediated AMR [1, 130, 150]. The difference in susceptibility between Ecl8Δ*ramR*<*acrAB*>Km and Ecl8<*acrAB*>Km may be because basal *ramA* expression from the Ecl8 background does not significantly impact on *acrAB* expression, hence no decrease in susceptibility. However, deletion of *acrAB* in a *ramA* overexpressing background should impact on susceptibility, consistent with our findings where Ecl8Δ*ramR*<*acrAB*>Km is more susceptible to PxB and colistin than Ecl8Δ*ramR*.

Ecl8<*tolC*>Km (MIC = 0.125 mg/L) was more susceptible to colistin than Ecl8 with a 1.5-fold lower MIC. However, in response to PxB, MIC values of the two strains were the same. Ecl8Δ*ramR*<*tolC*>Km PxB and colistin MICs did not differ from Ecl8Δ*ramR* or Ecl8<*ramR*>Km, indicating that *tolC* is not an important component of the RamA-mediated PxB and colistin resistance phenotype. This could be because *ramA* overexpression upregulates other resistance genes, such as *acrAB*, which compensate for *tolC* loss and retain the *ramA* overexpression phenotype. In the case of the Δ*ompC* and Δ*ompF* mutants, no differences were seen in MICs compared to corresponding Ecl8 or Ecl8Δ*ramR* controls, indicating no role for these porins in PxB or colistin resistance.

5.3.2. Characterising regulation of *lpxO* overexpression and its impact on tigecycline, PxB and colistin susceptibility

Specific LpxO-mediated lipid A modifications are associated with reduced susceptibility to colistin and CAMPs [183, 211]. The *lpxO* gene has been shown to be under the regulatory control of both the RamA transcription regulator and the PhoPQ TCS [1, 183, 209, 211]; the overexpression of either of these regulatory factors has also been linked to reduced polymyxin and CAMP susceptibility. RamA is also responsible for mediating a reduced susceptibility to tigecycline [134, 135, 143], with RamA-regulated genes such as *acrAB* playing a key role, as reported

previously and observed in this study [227, 229, 236]. As a result, we sought to define what role *lpxO* overexpression plays in RamA-mediated responses to polymyxins and tigecycline; is it able to independently reduce polymyxin susceptibility, and does it feature significantly in reducing susceptibility to tigecycline.

In order to assess the contribution of *lpxO* overexpression, we tested Ecl8/pAC*lpxO*, Ecl8Δ*ramR*/pAC*lpxO*, Ecl8/pBR*lpxO* and Ecl8Δ*ramR*/pBR*lpxO*, as well as the corresponding vector-only controls. We also tested the putative Δ*lpxO* mutants Ecl8<*lpxO*>Km and Ecl8Δ*ramR*<*lpxO*>Km; this was a chance to phenotypically validate these putative Δ*lpxO* mutants because PCR screens appeared to confirm presence of the *lpxO* gene (Figure 8). As putative Δ*lpxO* mutants, we would expect to see *lpxO* loss lead to an increase in susceptibility to polymyxins, as LpxO-mediated lipid A modifications reduce susceptibility to colistin and CAMPs [183, 211].

5.3.2.1. Overexpression of *lpxO* reduces tigecycline susceptibility

The results of strains overexpressing *lpxO* were mixed (Figure 9; Table 6). The MIC of Ecl8/pAC*lpxO* (MIC = 2 mg/L) was ten-fold that of Ecl8 (MIC = 0.25 mg/L). However, this MIC value was identical to that of the Ecl8/pACYC184 vector-only control (MIC = 2mg/L), indicating that the reduced susceptibility was associated with the plasmid, as opposed to overexpressed *lpxO*. Ecl8Δ*ramR*/pAC*lpxO* on the other hand demonstrated an MIC value (MIC = 4 mg/L) double that of Ecl8Δ*ramR* (MIC = 2 mg/L) and the Ecl8Δ*ramR*/pACYC184 control (MIC = 2 mg/L), suggesting that overexpressed *lpxO* elevates the RamA-mediated response to tigecycline in *K. pneumoniae*.

Ecl8/pBR*lpxO* showed a slight reduction in susceptibility to tigecycline compared to Ecl8 (1.5-fold); however Ecl8/pBR322 demonstrated an even greater decrease (4-fold). This suggests that reduced susceptibility was conferred by pBR322, not *lpxO* overexpression. In support of this, neither Ecl8Δ*ramR*/pBR*lpxO* nor the Ecl8Δ*ramR*/pBR322 control displayed any reduction in susceptibility compared to Ecl8Δ*ramR*.

The original putative Δ*lpxO* mutants, Ecl8<*lpxO*>Km and Ecl8Δ*ramR*<*lpxO*>Km, showed a contrastingly high level of resistance compared to any other strain tested except the similarly resistant Ecl8Δ*ramR*/pAC*lpxO* (Figure 9; Table 6). This was unexpected due to previous studies showing that decreased antimicrobial

susceptibility results from LpxO-mediated lipid A modifications [183, 211]. However, these observations were based on susceptibility to CAMPs and not tigecycline, therefore the role of *lpxO* deletion on tigecycline resistance may be different. LpxO-mediated lipid A modifications may potentially contribute to antimicrobial susceptibility differently in an antimicrobial-dependent manner. Nonetheless, the similar MICs of *lpxO* overexpressing Ecl8Δ*ramR*/pAC*lpxO*, and the Δ*lpxO* mutants – Ecl8<*lpxO*>Km and Ecl8Δ*ramR*<*lpxO*>Km – suggest that these mutants were in fact not successfully generated, as indicated by the original PCR validation (Figure 8).

5.3.2.2. Characterisation of the role of *lpxO* overexpression in polymyxin resistance is inconclusive

Overexpression of *lpxO* generated by the pAC*lpxO* construct in Ecl8 and Ecl8Δ*ramR* did not appear to reduce susceptibility to either PxB or colistin (Figure 10; Table 6). PxB MICs were the same between Ecl8/pAC*lpxO* and Ecl8 (MIC = 0.1875 mg/L for both), and Ecl8Δ*ramR*/pAC*lpxO* and Ecl8Δ*ramR* (MIC = 0.25 mg/L for both). The MIC of the Ecl8/pACYC184 control also corresponded to Ecl8/pAC*lpxO* and Ecl8; however, Ecl8Δ*ramR*/pACYC184 had a 1.3-fold lower MIC than Ecl8Δ*ramR*/pAC*lpxo* and Ecl8Δ*ramR*, suggesting that the vector might incur a fitness cost. Colistin MICs followed a similar pattern, where pAC*lpxO* conferred no reduction in susceptibility in Ecl8 and Ecl8Δ*ramR*, and the Ecl8Δ*ramR* /pACYC184 control had a 1.5-fold lower MIC than Ecl8Δ*ramR* /pAC*lpxO* and Ecl8Δ*ramR* (Figure 10; Table 6).

RSA results showed that, with one exception, pAC*lpxO* strains did not reduce susceptibility to PxB or colistin (Figures 13 and 14). The exception was Ecl8/pAC*lpxO*, which displayed statistically significant survival after exposure to 0.033 µg/ml of colistin (53.86 %) (Figure 14). Ecl8/pAC*lpxO* survival at 0.033 µg/ml was greater than both the Ecl8/pACYC184 control (0.82%) and the statistically insignificant survival of w/t Ecl8 (1.75%). In contrast Ecl8Δ*ramR*/pAC*lpxO,* though statistically significant, only displayed a survival percentage of 0.6 % which was 11-fold lower than that of Ecl8Δ*ramR* (6.74%). At higher concentrations of PxB and colistin pAC*lpxO* and pACYC184 strains were unable to survive, except for the statistically significant survival of the *ramA* overexpressing Ecl8Δ*ramR*/pACYC184 at 0.096 µg/ml of PxB (0.24%) (Figure 13). These RSA results are inconclusive; the greater survival of Ecl8/pAC*lpxO* compared to Ecl8Δ*ramR*/pAC*lpxO* suggests that *ramA* overexpression does not reduce colistin susceptibility. However, this is

possibly an anomaly, as we have demonstrated that *ramA* overexpression increases both PxB and colistin MICs.

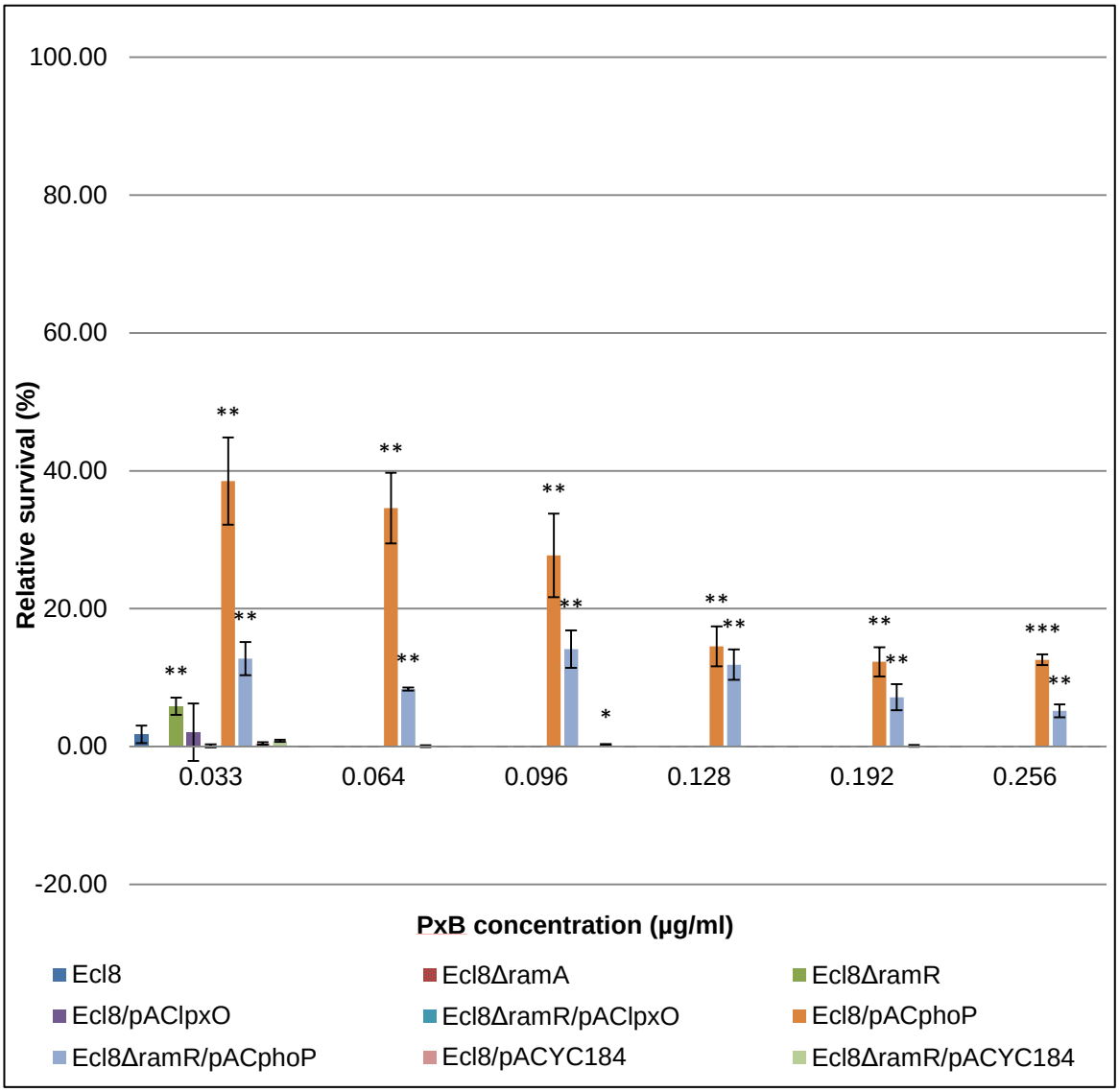

Figure 13. Relative survival assay of *K. pneumoniae* (Ecl8 w/t, mutant, pAC*lpxO*, pAC*phoP*, pACYC184 strains) to PxB. Bacteria were incubated stationary, with or without PxB, for 1 hour at 37°C before being serially diluted and 50 µl spread on LB agar half-plates in duplicate. After overnight incubation at 37°C, single colonies were counted and CFU/ml calculated to 10^5. The relative survival was calculated as a percentage of surviving exposed bacteria compared to corresponding unexposed bacteria. Error bars show the standard deviation of the relative survival percentages derived from the replicates. *Student's T-test* was performed on samples at each antibiotic concentration to determine if the CFU/ml was statistically significant compared to the w/t Ecl8 control at the same concentration: * for p <0.05, ** for p <0.005, *** for p <0.0005. Results show data from one experiment, performed in quadruplicate.

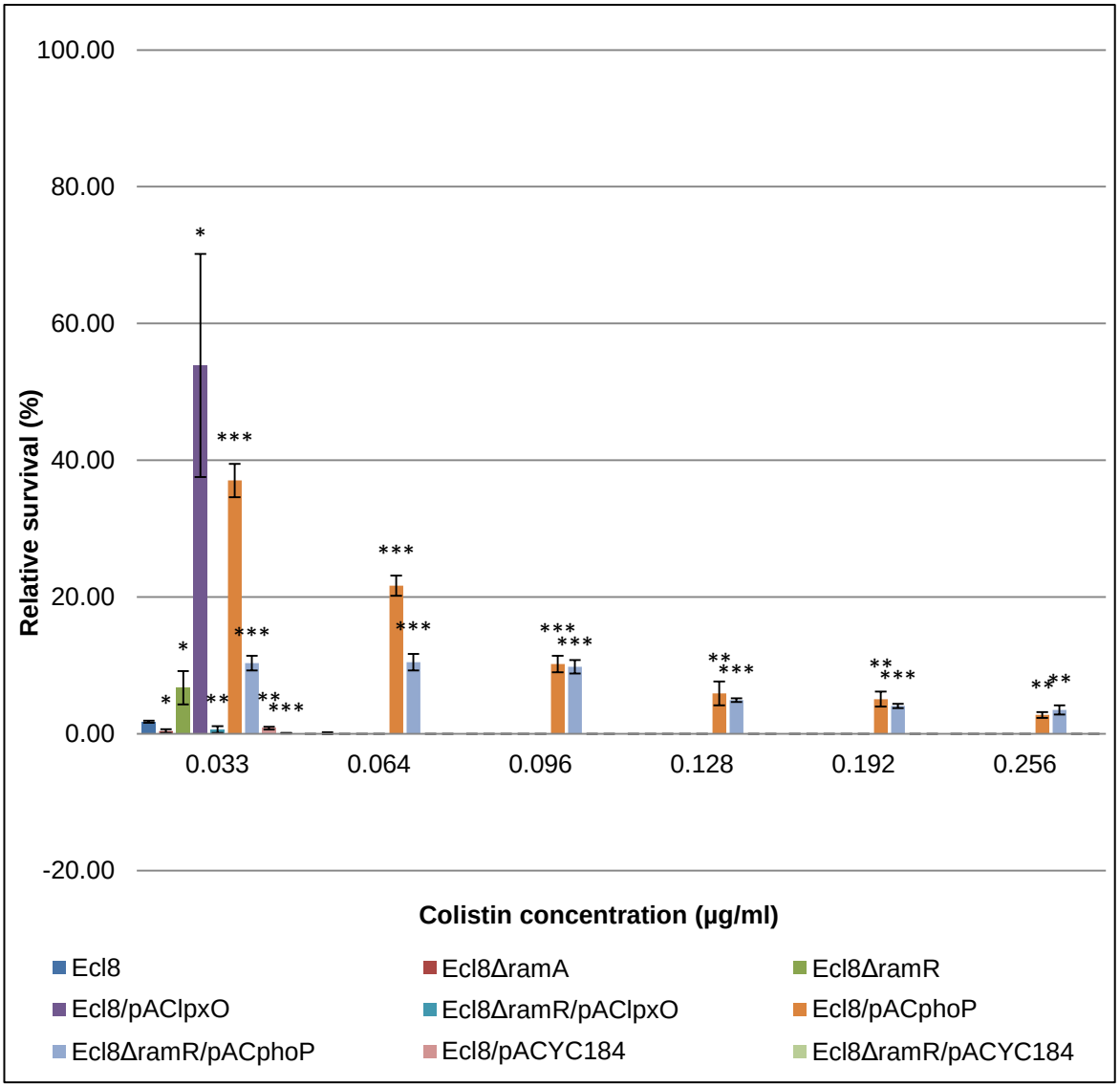

Figure 14. Relative survival assay of *K. pneumoniae* (Ecl8 w/t, mutant, pAC*lpxO*, pAC*phoP*, pACYC184 strains) to colistin. Bacteria were incubated stationary, with or without colistin, for 1 hour at 37°C before being serially diluted and 50 µl spread on LB agar half-plates in duplicate. After overnight incubation at 37°C, single colonies were counted and CFU/ml calculated to 10^5. The relative survival was calculated as a percentage of surviving exposed bacteria compared to corresponding unexposed bacteria. Error bars show the standard deviation of the relative survival percentages derived from the replicates. *Student's T-test* was performed on samples at each antibiotic concentration to determine if the CFU/ml was statistically significant compared to the w/t Ecl8 control at the same concentration: * for p <0.05, ** for p <0.005, *** for p <0.0005. Results show data from one experiment, performed in quadruplicate.

Despite the higher copy number of the pBR322 construct, the PxB MIC value of Ecl8/pBR*lpxO* (MIC = 0.125 mg/L) was 1.5-fold lower than w/t Ecl8, and the Ecl8Δ*ramR*/pBR*lpxO* PxB MIC (MIC = 0.1875 mg/L) was 1.3-fold lower than Ecl8Δ*ramR* (Figure 10; Table 6). Interestingly, the MIC of Ecl8/pBR322 (MIC = 0.25 mg/L) was 2-fold higher than Ecl8/pBR*lpxO* and Ecl8, whilst contrastingly Ecl8Δ*ramR*/pBR322 (MIC = 0.1875 mg/L) was 1.3-fold more susceptible to PxB than Ecl8Δ*ramR*. Similarly, pBR*lpxO* did not reduce susceptibility to colistin in Ecl8 or Ecl8Δ*ramR*, whilst the Ecl8Δ*ramR*/pBR322 control was again more susceptible than Ecl8Δ*ramR*/pBR*lpxO* and Ecl8Δ*ramR* (1.5-fold). The case may be that pBR322 is detrimental to the bacterium's ability to grow, having a knock-on effect on the pBR*lpxO* construct and thereby affecting its performance in our antimicrobial susceptibility assays.

We also tested the putative Δ*lpxO* strains Ecl8<*lpxO*>Km and Ecl8Δ*ramR*<*lpxO*>km, finding that MIC values for PxB and colistin, were much greater than those for Ecl8 and Ecl8Δ*ramR* respectively (Figure 10; Table 6). This forms a pattern with the Ecl8<*lpxO*>Km and Ecl8Δ*ramR*<*lpxO*>km results from tigecycline exposure, where unexpectedly high MICs were also observed (Figure 9; Table 6), and reinforces the observation that these putative mutants were not successfully generated.

5.3.3. Characterisation of overexpression constructs in *E. coli* DH10β

In order to transform our plasmid constructs into Ecl8 and Ecl8Δ*ramR*, they first had to be transformed into *E. coli* DH10β. These construct-bearing strains were also exposed to tigecycline, PxB and colistin with MICs determined to test if our plasmid constructs could confer resistance comparable to *K. pneumoniae* w/t Ecl8, Ecl8Δ*ramA* or Ecl8Δ*ramR* strains.

5.3.3.1. pAC*lpxO* reduces susceptibility of *E. coli* DH10β to tigecycline

The DH10β strains containing pAC*ramA* and pACYC177 constructs both presented tigecycline MICs of 0.125 mg/L, suggesting that pAC*ramA*-mediated *ramA* overexpression did not affect tigecycline susceptibility (Figure 15; Table 7). However, we have demonstrated that Ecl8Δ*ramR* plays a major role in reducing tigecycline susceptibility, consistent with other reports [134, 135, 143], thus suggesting that the pAC*ramA* construct is erroneous. Only DH10β/pAC*lpxO* (MIC = 0.375 mg/L) demonstrated a reduction in tigecycline susceptibility compared to its

control, with a 3-fold greater MIC relative to DH10β/pACYC184 (MIC = 0.125 mg/L), and roughly 2-fold greater than Ecl8, Ecl8Δ*ramA*, Ecl8<*ramA*>Km and Ecl8<*romAramA*>Km (Figure 15; Table 7). This indicates a potential role for *lpxO* expression in reducing susceptibility to tigecycline, consistent with the high MIC of Ecl8Δ*ramR*/pAC*lpxO*. The absence of a DH10β/pBR322 control prevents a full assessment of how DH10β/pBR*lpxO* affects susceptibly; however its 1.5-fold greater MIC value (MIC = 0.1875 mg/L) compared to DH10β/pAC*ramA*, DH10β/pACYC177 and DH10β/pACYC184 (MIC = 0.125 mg/L for all) supports the implication of *lpxO* overexpression in reduced tigecycline susceptibility.

However, when compared with Ecl8<*ramR*>Km and Ecl8Δ*ramR*, the MIC of DH10β/pAC*lpxO* was 5.3-fold lower, and DH10β/pBR*lpxO* 10.6-fold lower. These observations, allied to the low MICs of DH10β/pAC*ramA*, DH10β/pACYC177 and DH10β/pACYC184 – at least 1.5-fold lower than MICS of Ecl8, Ecl8Δ*ramA*, Ecl8<*ramA*>Km or Ecl8<*romAramA*>Km – may have several reasons. *E. coli* may be inherently more susceptible to tigecycline compared to *K. pneumoniae*, or alternatively the introduction of the plasmids into DH10β may confer a fitness cost, affecting the organism's growth and ability to resist in spite of the overexpression of resistance-associated genes [234]. Another reason for their ineffectiveness may be that the plasmid constructs, whilst phenotypically correct when generated, may not be successful gene overexpression constructs. An error in the plasmid sequence, for example, may result in lower amounts of gene product than expected. This theory is supported by the ineffectiveness of pAC*ramA* in reducing susceptibility to tigecycline, despite our results showing that chromosomal *ramA* expression in Ecl8Δ*ramR* and Ecl8<*ramR*>Km reduces susceptibility substantially.

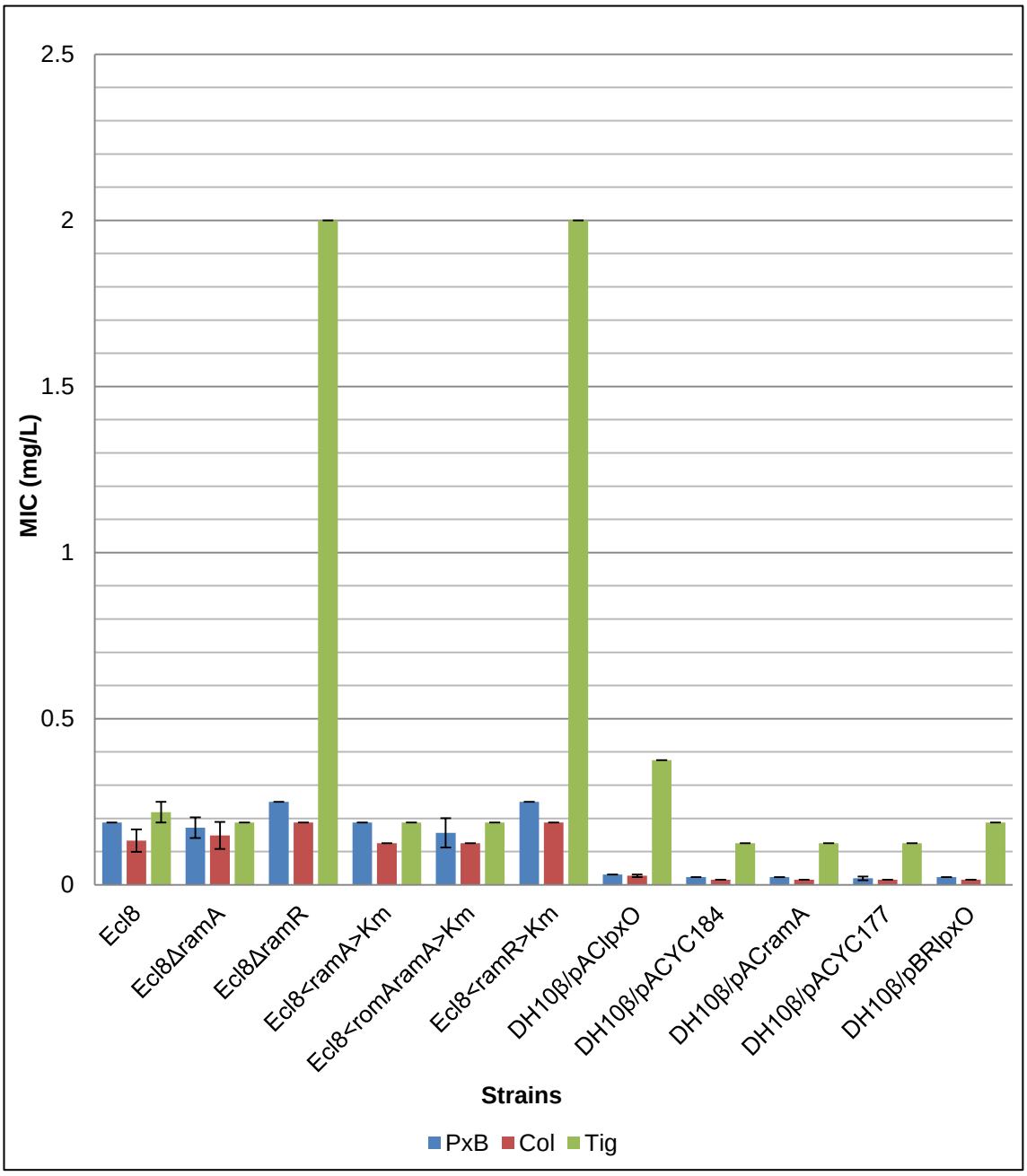

Figure 15. MIC of *K. pneumoniae* Ecl8 w/t and mutant strains and *E. coli* DH10β plasmid constructs to PxB, colistin and tigecycline. MICs are presented as an average of the experimentally derived results. Each strain was tested at least once, in duplicate. Error bars show the standard deviation of samples' MIC values.

Table 7. MIC of *K. pneumoniae* Ecl8 w/t and mutant strains and *E. coli* DH10β plasmid constructs to PxB, colistin and tigecycline.

Strains	PxB (mg/L)	Colistin (mg/L)	Tigecycline (mg/L)
Ecl8	0.1875[2]	0.094 – 0.1875[2]	0.1875 – 0.25[2]
Ecl8Δ*ramA*	0.125 – 0.1875[2]	0.094 – 0.1875[2]	0.1875[2]
Ecl8Δ*ramR*	0.25[2]	0.1875[2]	2[2]
Ecl8<*ramA*>Km	0.1875[1]	0.125[1]	0.1875[1]
Ecl8<*romAramA*>Km	0.125 – 0.1875[1]	0.125[1]	0.1875[1]
Ecl8<*ramR*>Km	0.25[1]	0.1875[1]	2[1]
DH10β/pAC*lpxO*	0.0312[1]	0.0234 – 0.0312[1]	0.375[1]
DH10β/pACYC184	0.0234[1]	0.0156[1]	0.125[1]
DH10β/pAC*ramA*	0.0234[1]	0.0156[1]	0.125[1]
DH10β/pACYC177	0.0156 – 0.0234[1]	0.0156[1]	0.125[1]
DH10β/pBR*lpxO*	0.0234[1]	0.0156[1]	0.1875[1]

Table 7. Each strain was tested at least once, in duplicate. For in-text referencing of MIC values, the higher MIC is used. [1] Strains were tested once, in duplicate; [2] strains were tested twice, each time in duplicate; [3] strains were tested on three occasions, each time in duplicate.

5.3.3.2. pAC*lpxO* reduces PxB and colistin susceptibility in *E. coli* DH10β

pAC*ramA* failed to mediate a reduction in susceptibility to PxB and colistin when introduced into *E. coli* DH10β compared to the corresponding vector-only control

(Figure 15; Table 7). DH10β/pBR*lpxO* also failed to reduce susceptibility compared to DH10β/pAC*ramA*, DH10β/pACYC177 and DH10β/pACYC184, though without a DH10β/pBR322 control we cannot draw any definitive conclusion as to its impact on susceptibility. In contrast, DH10β/pAC*lpxO* (PxB and colistin MIC = 0.0312 mg/L) conferred a minor reduction in susceptibility compared to the DH10β/pACYC184 control (PxB MIC = 0.0234 mg/L; colistin MIC = 0.0156 mg/L), implicating *lpxO* expression as an important contributor to this phenotype.

Whilst the apparent ineffectiveness of pAC*ramA and pBRlpxO* may be due to faults in their construction, it may alternatively support the idea that DH10β is considerably more susceptible to these antimicrobials than *K. pneumoniae*. Our MIC and RSA results show that plasmid-mediated *phoP* overexpression in *K. pneumoniae* plays an important role in reducing susceptibility to PxB and colistin (Figures 10, 13 and 14; Table 6). Time permitting; it would therefore be interesting to assess the effect of pAC*phoP* introduction into DH10β; to act as a control for the other plasmid constructs to investigate if DH10β/pAC*phoP* is able to mediate a reduction in polymyxin susceptibility similar to that seen in *K. pneumoniae*.

5.3.4. Characterisation of the role of *phoP* overexpression in tigecycline, PxB and colistin resistance

The PhoPQ TCS plays an important role in promoting resistance in the face of challenge by polymyxins and host CAMPs [177, 183, 196-199, 211]. Of the PhoPQ TCS, *phoP* has been reported to be the driver of CAMP and polymyxin resistance when constitutively activated [198, 203, 204]. We therefore performed MIC and RSA experiments to characterise the role of *phoP* overexpression in tigecycline and polymyxin susceptibility; comparing its contribution to *lpxO* and *ramA* overexpression, and determining any correlation between *lpxO*- and *phoP*-mediated phenotypes that might indicate a role for PhoPQ regulation of *lpxO*. Ecl8/pAC*phoP* and Ecl8Δ*ramR*/pAC*phoP* were included to assess whether increased *phoP* overexpression reduces susceptibility in w/t *K. pneumoniae*, or is able to further reduce susceptibility in *ramA*-overexpressing strains. Ideally, had their generation been successful, we would also have included Δ*lpxO* and Δ*phoP* mutants to compare how their inactivation affected susceptibility. We conducted tigecycline MIC experiments in fulfilment of our aim to characterise the role of *ramA* and RamA-regulated permeability genes in reduced susceptibility to this drug. Despite a

literature search revealing no previously reported role for *phoPQ* overexpression in reduced tigecycline susceptibility, we also tested pAC*phoP* strains to examine any potential role that *phoP* overexpression may play in this phenotype.

5.3.4.1. Overexpression of *phoP* does not reduce tigecycline susceptibility

To assess any potential role that *phoP* may play in RamA-mediated tigecycline resistance, *phoP*-overexpressing Ecl8 and Ecl8Δ*ramR* were exposed to varying concentrations of tigecycline (Figure 9; Table 6). Although Ecl8/pAC*phoP* shows an 8-fold higher MIC (MIC = 2 mg/L) than w/t Ecl8 (MIC = 0.25 mg/L), the corresponding Ecl8/pACYC184 control also shows the same value (MIC = 2 mg/L), suggesting that the increased resistance is conferred by the plasmid, not the plasmid-mediated overexpression of *phoP*. In Ecl8Δ*ramR*/pAC*phoP* there is no increase in resistance compared to Ecl8Δ*ramR*, whilst the Ecl8Δ*ramR*/pACYC184 control also shows the same value (MIC = 2 mg/L). Based on these results, we inferred that in *K. pneumoniae*, *phoP* does not have a measurable impact on tigecycline resistance.

5.3.4.2. Overexpression of *phoP* drives polymyxin resistance

Previous studies have reported that PhoPQ mediates resistance to PxB and colistin in *K. pneumoniae* by activating genes associated with lipid A modification [183, 196-199, 211]. We show that *phoP* overexpression substantially increases resistance to both PxB and colistin independently of *ramA* or *lpxO*. For both PxB and colistin, Ecl8/pAC*phoP* and Ecl8Δ*ramR*/pAC*phoP* produced much higher MIC values than Ecl8 and Ecl8Δ*ramR* respectively, as well as pAC*lpxO*- and pAC*ramA*-producing strains, and the corresponding pACYC184 controls (Figure 10; Table 6). For PxB, Ecl8/pAC*phoP* produced a 5-fold higher MIC (MIC = 1 mg/L) than Ecl8 (MIC = 0.1875 mg/L), whilst the MIC of Ecl8Δ*ramR*/pAC*phoP* (MIC = 1 mg/L) was 4-fold higher than Ecl8Δ*ramR* (MIC = 0.25 mg/L). Similarly, in response to colistin, the MIC of Ecl8/pAC*phoP* (MIC = 0.5 mg/L) was 2.6-fold that of Ecl8 (MIC = 0.1875 mg/L), whilst Ecl8Δ*ramR*/pAC*phoP* (MIC = 1 mg/L) was 5-fold that of Ecl8Δ*ramR* (MIC = 0.1875 mg/L).

We also tested various existing *S.* Typhimurium knockout strains, where genes including *ramR*, *ramA*, *phoP* and *phoPQ* were inactivated (Figure 16; Table 8). In *Salmonella*, *ramA* and *phoPQ* have both been associated with MDR [125, 128, 129, 177, 200]. We wanted to determine if a *S.* Typhimurium Δ*ramR* mutant conferred a

similar susceptibility trend to corresponding *K. pneumoniae* Δ*ramR* mutants. Comparisons between *S*. Typhimurium w/t 4/74 and 4/74Δ*ramR* showed that *ramA* overexpression did not reduce susceptibility to PxB and colistin, as occurs with *ramA* overexpression in *K. pneumoniae* Ecl8. In fact, in 4/74Δ*ramRA*, the absence of the *ram* operon did not increase susceptibility. We also wanted to assess the impact of *phoP* and *phoPQ* loss on *S*. Typhimurium polymyxin susceptibility. *S.* Typhimurium 4/74Δ*phoP*Δ*ramR*, 4/74Δ*phoP*Δ*ramRA*, and 4/74Δ*phoPQ* (PxB MIC = 0.0468 mg/L for all; colistin MIC = 0.0234 mg/L for all) all demonstrated a 4-fold increase in susceptibility compared to w/t *S.* Typhimurium (PxB and colistin MIC = 0.1875 mg/L), and a minimum 5.3-fold increase compared to the tested *K. pneumoniae* strains, indicating the importance of *phoP* in mediating survival. Ideally we would also test *K. pneumoniae* Δ*phoPQ* mutants, instead of relying on interpretations about the importance of *phoPQ* from *Salmonella*; however, generating these mutants proved unsuccessful. Because *phoPQ* has been shown to be important in *K. pneumoniae* AMR [183, 211], as it is in *Salmonella* [177, 200], interpretations concerning the significance of PhoPQ are likely to be consistent between these two species.

The PxB and colistin RSAs confirm that of the various genes we tested, *phoP* was the key driver of resistance to these agents (Figures 13 and 14). Despite several strains exhibiting survival at 0.033 µg/ml of PxB and colistin, the only strains showing statistically significant survival up to and including the highest concentrations tested were Ecl8/pAC*phoP* and Ecl8Δ*ramR*/pAC*phoP*. The corresponding pACYC184 controls showed no survival at high antibiotic concentrations, bar the survival of Ecl8Δ*ramR*/pACYC184 at 0.096 µg/ml of PxB, signifying that the pAC*phoP* resistance phenotypes were *phoP*-dependent and not an influence of the vector. This indicates that, of the various genes we tested, resistance to PxB and colistin is primarily influenced by *phoP*. Interestingly, of Ecl8/pAC*phoP* and Ecl8Δ*ramR*/pAC*phoP*, the former demonstrates consistently higher survival across increasing PxB and colistin concentrations, despite the absence of *ramA* overexpression. These results therefore suggest that *ramA* overexpression is not significant in mediating PxB and colistin resistance compared to *phoP* overexpression. This observation is backed up by the inability of other *ramA* overexpressing strains to survive at higher antibiotic concentrations. This contrasts with the previously reported role of *ramA* overexpression in reducing PxB and

colistin susceptibility [1], and might represent a more dominant role for PhoPQ regulation in this phenotype.

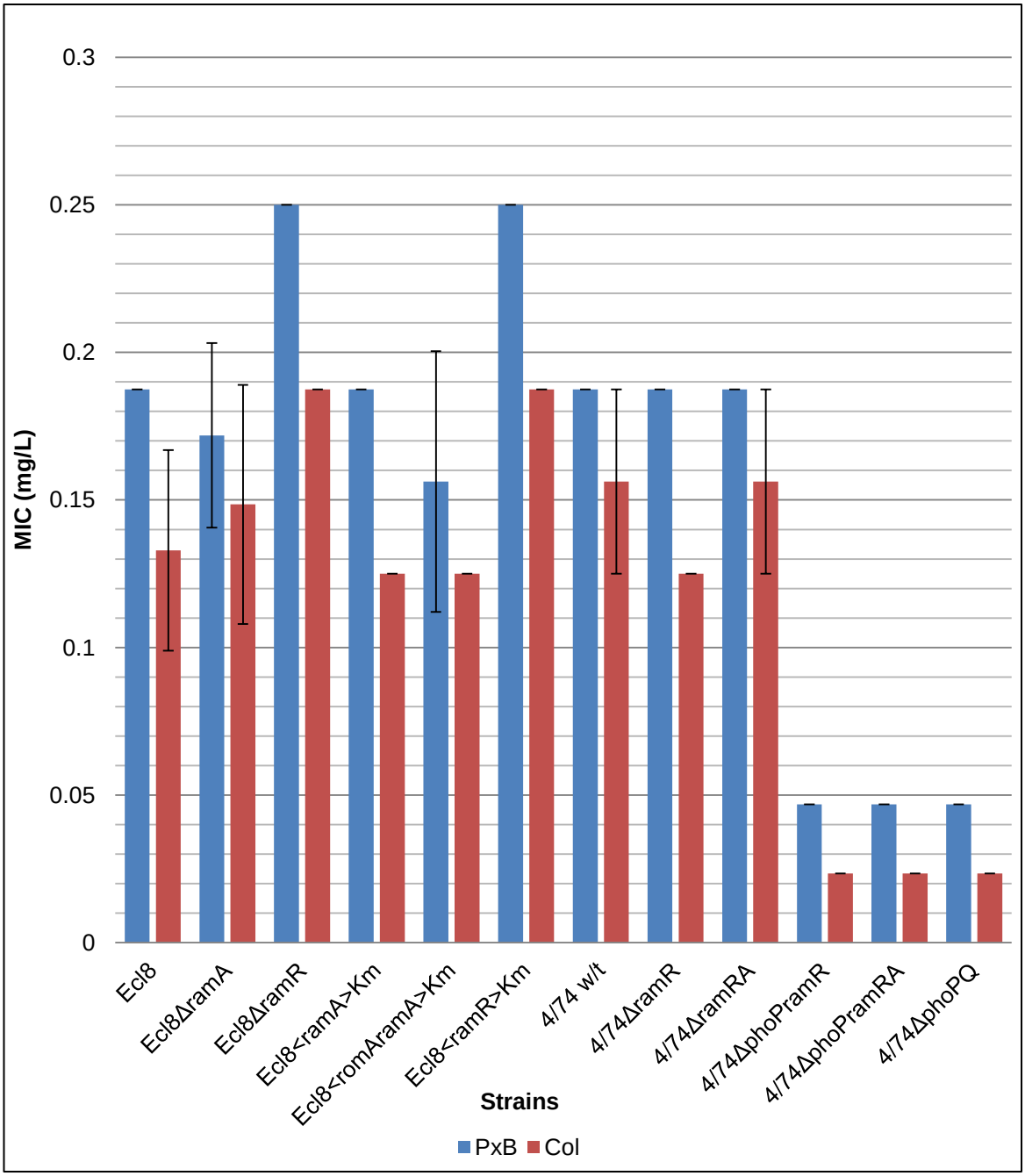

Figure 16. MIC of *K. pneumoniae* Ecl8 w/t and mutant strains and *S.* Typhimurium 4/74 w/t and mutant strains to PxB and colistin. MICs are presented as an average of the experimentally derived results. Each strain was tested at least once, in duplicate. Error bars show the standard deviation of samples' MIC values.

Table 8. MIC of *K. pneumoniae* Ecl8 w/t and mutant strains and *S.* Typhimurium 4/74 w/t and mutant strains to PxB and colistin.

Strains	PxB (mg/L)	Colistin (mg/L)
Ecl8	0.1875[2]	0.094 – 0.1875[2]
Ecl8Δ*ramA*	0.125 – 0.1875[2]	0.094 – 0.1875[2]
Ecl8Δ*ramR*	0.25[2]	0.1875[2]
Ecl8<*ramA*>Km	0.1875[1]	0.125[1]
Ecl8<*romAramA*>Km	0.125 – 0.1875[1]	0.125[1]
Ecl8<*ramR*>Km	0.25[1]	0.1875[1]
4/74 w/t	0.1875[1]	0.125 – 0.1875[1]
4/74Δ*ramR*	0.1875[1]	0.125[1]
4/74Δ*ramRA*	0.1875[1]	0.125 – 0.1875[1]
4/74Δ*phoPramR*	0.0468[1]	0.0234[1]
4/74Δ*phoPramRA*	0.0468[1]	0.0234[1]
4/74Δ*phoPQ*	0.0468[1]	0.0234[1]

Table 8. Each strain was tested at least once, in duplicate. For in-text referencing of MIC values, the higher MIC is used. [1] Strains were tested once, in duplicate; [2] strains were tested twice, each time in duplicate; [3] strains were tested on three occasions, each time in duplicate.

6. Discussion

K. pneumoniae is an increasingly important pathogen worldwide due to the rapid emergence and dissemination of MDR strains. MDR *K. pneumoniae* infection has severe ramifications, and is particularly threatening to vulnerable patients, such as the elderly or immunocompromised [45]. Recently, there has been an increasing interest in the use of the last-line drugs tigecycline and colistin for the treatment of MDR *K. pneumoniae* infection [80, 144-146]. However, resistance to even these last-line antimicrobials has begun to emerge, with intrinsic bacterial factors such as the RamA and PhoPQ regulators playing a major role [1, 134, 135, 143, 177, 183, 196-199, 211]. In this report, we have assessed how overexpression of *ramA*, *phoP* and *lpxO* genes contributes to tigecycline, PxB and colistin susceptibility. We also assessed the role of *K. pneumoniae* RamA regulation in susceptibility to these drugs via inactivation of *ramA* and RamA-regulated permeability genes: *acrAB, tolC*, o*mpC* and o*mpF*. We were unable to generate *phoPQ* knockout mutants therefore we examined the impact of PhoPQ regulation on polymyxin susceptibility using pre-existing *S*. Typhimurium *phoPQ* knockouts.

Our findings corroborate with previous studies where *ramA* overexpression is responsible for reduced tigecycline susceptibility in *K. pneumoniae* [134, 135, 143, 238], with important roles for RamA-regulated permeability components, including the efflux pump AcrAB and TolC and the OM porin OmpF (Figure 9; Table 6). We report that chromosomal *ramA* overexpression mediated by *ramR* inactivation reduces susceptibility to tigecycline. We also show that inactivation of *acrAB* and *tolC* efflux genes increases susceptibility, consistent with reports of tigecycline resistance being mediated by the AcrAB-TolC efflux pump [227, 228, 235-238]. We also observed that inactivation of *ompF* led to an increased tigecycline MIC. However, a literature search failed to identify previous reports where inactivated *ompF* was responsible for tigecycline resistance. It has been suggested that tigecycline influx into the bacterial cell is in part mediated by the porins OmpF and OmpC [239], similar to other tetracyclines [105, 240], and that decreased *ompF* levels contribute to reduced tetracycline susceptibility [118, 241]. Linkevicius et al. [239] describe how gene mutations in *E. coli* affecting LPS heptose biosynthesis, which can result in decreased porin expression, leads to reduced tigecycline susceptibility. Alterations to LPS, OM stability and porin activity that prevent tigecycline influx may consequently limit the drug's activity and reduce sensitivity. This mechanism of reduced cell entry would corroborate our findings, where loss of

ompF may prevent tigecycline influx, thereby resulting in the reduced tigecycline susceptibility phenotype shown here. In contrast, no effects on tigecycline susceptibility were seen in *ompC* mutants compared to controls. Due to the similar tigecycline MICs of *acrAB* and *tolC* knockout mutants, we have not managed to shed light on which of these genes contributes most to tigecycline susceptibility. It would therefore be interesting to assess the effect of *acrAB* and *tolC* overexpression in an Ecl8Δ*ramA* background; to observe whether *tolC* or *acrAB* overexpression can reduce susceptibility to tigecycline in the absence of *ramA*. These potential future experiments would help to define whether *acrAB* and *tolC* are key components of RamA-mediated tigecycline resistance.

We further demonstrate that overexpressed *phoP* appears to have no role in increasing tigecycline resistance (Figure 9; Table 6). On the other hand, plasmid-mediated *lpxO* overexpression in *K. pneumoniae* Ecl8Δ*ramR* exhibited a 2-fold higher tigecycline MIC than Ecl8Δ*ramR* alone, or the vector-only control. However, this effect seems dependent on chromosomal *ramA* overexpression, as Ecl8/pAC*lpxO* displayed no susceptibility changes compared to w/t Ecl8 and Ecl8/pACYC184. Additionally, in *E. coli* DH10β, introduction of pAC*lpxO* conferred a 3-fold and 2-fold reduction in susceptibility compared to the vector-only control and w/t Ecl8 respectively (Figure 15; Table 7), implicating *lpxO* overexpression as a factor in tigecycline susceptibility. Tigecycline resistance is typically mediated by increased activity of efflux pumps such as AcrAB [227, 228, 235-238], with no studies found that reported a role for LpxO or other lipid A modifiers. Tigecycline is a derivative of the tetracycline class of antimicrobials; these drugs can cross the OM via porins as a cationic complex [240]. Therefore lipid A modifications, which neutralise LPS charge and increase OM stability [105, 176, 177, 191], may prevent the ability of tigecycline to bind and traverse the OM, perhaps explaining why *lpxO* overexpression reduced tigecycline sensitivity. The effect of *lpxO* overexpression on reducing tigecycline susceptibility is primarily seen in Ecl8Δ*ramR* as opposed to Ecl8; this may be explained by the increased efflux and decreased influx mediated by *ramA* overexpression, which we show also contributes to tigecycline resistance, and which may amplify the effects of *lpxO* expression.

The chromosomal *ramA* overexpressors Ecl8Δ*ramR* and Ecl8<*ramR*>Km both demonstrated reduced susceptibility to PxB and colistin compared to w/t Ecl8 (Figure 10; Table 6), thus confirming that *ramA* overexpression reduces

susceptibility to PxB and colistin. Of the RamA-regulated genes tested, Ecl8Δ*ramR*<*acrAB*>Km displayed increased susceptibility to PxB and colistin, indicating that *acrAB* is an important component of RamA-mediated polymyxin resistance (Figure 10; Table 6). This corresponds to a previous report where AcrAB was shown to contribute to CAMP resistance [216]. Inactivation of *tolC* increased susceptibility to colistin only in Ecl8; this may indicate that *ramA* overexpression compensates for loss of *tolC* by increasing expression of other resistance genes, such as *acrAB*, thereby maintaining the polymyxin resistance phenotype. We did not detect any difference in susceptibility with *ompF* or *ompC* knocked out in either a w/t or *ramA* overexpressing background, indicating that porins play no part in RamA-mediated polymyxin resistance (Figure 10; Table 6).

The impact of *lpxO* overexpression on polymyxin susceptibility was inconclusive, with pAC*lpxO* and pBR*lpxO* in w/t and *ramA*-overexpressing backgrounds unable to reduce susceptibility in MIC experiments compared to w/t Ecl8 and Ecl8Δ*ramR* (Figure 10; Table 6). Contrastingly, in *E. coli* DH10β pAC*lpxO* increased PxB and colistin MICs, with DH10β/pAC*lpxO* conferring a minor decrease in susceptibility to both agents compared to the vector-only control. Likewise, the colistin survival assay displayed a greater relative survival at 0.033 µg/ml for Ecl8/pAC*lpxO* compared to Ecl8 and the Ecl8/pACYC184 control (Figure 14). Interestingly, Ecl8Δ*ramR*/pAC*lpxO* exhibited lower survival at 0.033 µg/ml colistin than Ecl8/pAC*lpxO*. A similar colistin survival percentage between Ecl8/pAC*lpxO* and Ecl8Δ*ramR*/pAC*lpxO* might indicate that *lpxO* is important to *ramA*-mediated polymyxin resistance; however, we instead see the opposite, where *lpxO* overexpression mediates colistin survival where *ramA* overexpression does not. This was unexpected as PxB and colistin MICs show that *lpxO* overexpression in w/t and *ramA* overexpressing backgrounds does not reduce polymyxin susceptibility, in contrast to chromosomal *ramA* overexpression (Figure 10; Table 6). Methodological differences in the way in which MIC experiments and RSAs are performed may explain the different results observed, where the high survival percentage of Ecl8pAC*lpxO* does not correspond to the low pAC*lpxO* PxB and colistin MICs. Taken together, the role of *lpxO* overexpression in polymyxin resistance is unclear due to our conflicting results. Although other studies have linked *K. pneumoniae lpxO* with an important role in polymyxin and CAMP resistance [1, 183, 206], they did not assess the effects of *lpxO* overexpression in isolation, and have instead considered its role in the context of transcriptional

regulators that control multiple genes. These regulators may therefore mediate reduced polymyxin susceptibility via a variety of alternative mechanisms, with *lpxO* potentially playing an accessory role. As a result, repetition of antimicrobial susceptibility experiments is necessary to define whether LpxO-mediated lipid A modifications are significant to this phenotype; generating knockout *lpxO* mutants is important in order to characterise the functional phenotype resulting from gene loss; and antimicrobial binding and permeabilisation assays would be useful to assess if the activity of polymyxins or CAMPs is reduced by *lpxO* overexpression.

We report that *phoP* overexpression mediates PxB and colistin resistance independently of *ramA*, due to the greater MIC values of pAC*phoP* strains (ranging from 2.6-fold to 4-fold) (Figure10; Table 6) and greater survival at higher antibiotic concentrations (Figures 14 and 15) compared to Ecl8Δ*ramR* and Ecl8<*ramR*>Km. We also show that inactivation of the *phoPQ* locus in *S*. Typhimurium results in substantially increased polymyxin susceptibility (Figure 16; Table 8). PhoPQ regulates *lpxO* in *K. pneumoniae*, and subsequent LpxO-mediated lipid A modifications contribute to polymyxin and CAMP resistance [183, 206]; therefore we would expect to see similar polymyxin MIC values between *phoP* and *lpxO* overexpressing strains. Instead we see substantially higher PxB and colistin MICs for pAC*phoP* strains compared to pAC*lpxO* and pBR*lpxO* strains, which indicates that LpxO-mediated lipid A modifications are not essential to *phoP*-mediated polymyxin resistance. The observed role for *phoP* expression in *K. pneumoniae* polymyxin resistance is significant due to the potential impacts on treating infections. The potential for cross-resistance also presents a troublesome mechanism whereby polymyxins and host immune factors, such as human LL-37, are able to induce PhoPQ [183, 192, 211], resulting in cross-resistance to CAMPs. In our study both *phoP* and *ramA* overexpression reduced polymyxin susceptibility. Due to the previously reported impact of *ramA* overexpression on reducing both polymyxin and LL-37 susceptibility [1], the potential for the involvement of *ramA* in cross-resistance requires further investigation.

Problems were encountered throughout the project, impacting on time and preventing a more thorough investigation of the hypothesis. Therefore the next steps of this project must be to repeat MICs and RSAs in order to thoroughly characterise target genes, assess the consistency of our findings, and confirm or reject our conclusions. One such problem was generating *lpxO* and *phoPQ* knockout mutants,

in response to the finding that our original *lpxO* knockout mutants still retained the *lpxO* gene (Figure 8). Despite using an established protocol [217], we were repeatedly unable to genotypically confirm by PCR the absence of the *lpxO* gene, despite observing a Km^R phenotype indicating a successful integration of the replacement Km cassette into the chromosome. This may have been simply an issue with our primers; however replacement primers were purchased and tested with no success, and Km primers specific for the cassette were used by other laboratory members to confirm successful knockout of other genes using the same protocol. The reproducibility of protocols is often a problem in laboratories with increasing awareness in the scientific media [242, 243]. In our case, erroneous integration of the Km cassette or gene duplication events as environmental adaptations are possible occurrences in procedures involving manipulation of bacterial genes [244, 245], and may explain the retention of both the *lpxO* gene and a Km^R phenotype. Whilst we did not anticipate any gene duplication events due to previous reports of successful *lpxO* and *phoPQ* deletion [183, 206], the retention or duplication of *lpxO* may also explain the unexpectedly high MICs of our putative Δ*lpxO* mutants to all antimicrobials tested. Similar MICs were not observed with other knockout strains tested where the gene of interest was also replaced with a Km cassette, suggesting that the high MIC values of Ecl8<*lpxO*>Km and Ecl8Δ*ramR*<*lpxO*>Km are not an artefact of the Km cassette. The inability to generate mutants is unfortunate as assessing the impact of *lpxO* and *phoPQ* knockout on susceptibility to PxB and colistin, together with the effects of plasmid-mediated *lpxO* and *phoP* overexpression, would allow a much more thorough characterisation of their roles. As such, an important next step for this project will be to generate these mutants, possibly utilising a different approach in order to negotiate the difficulties faced with our exchange protocol.

Interpreting our findings was also difficult due to the inconsistent and unexpected outcomes of our plasmid constructs. For example, the pBR322 control appeared to drive a reduction in susceptibility to tigecycline instead of pBR*lpxO* (Figure 9; Table 6), whereas the introduction of pACYC184 into Ecl8Δ*ramR* increased susceptibility to colistin (Figure 10; Table 6). Furthermore, the inconsistent activity of the pAC*ramA*, pAC*lpxO* and pBR*lpxO* constructs might suggest that they were not generated correctly, or not transformed appropriately into Ecl8 and Ecl8Δ*ramR*. However, pAC*phoP*, constructed in much the same way, demonstrates a very clear

resistance phenotype to PxB and colistin incomparable to other tested strains. This indicates that the pAC*phoP* construct was successfully generated, and would imply that the other constructs were as well.

Interestingly, pACramA strains did not demonstrate reduced susceptibility to any of the antimicrobials tested in spite of the increased MICs of Ecl8Δ*ramR* and Ecl8<*ramR*>Km. Where pAC*ramA* strains did exhibit reduced susceptibility, observed in tigecycline MICs and PxB and colistin RSAs, this often corresponded to similar susceptibility of the pACYC177 vector-only controls, with the common theme among less susceptible pAC*ramA* and pACYC177 strains being an Ecl8Δ*ramR* background. This confirms that *ramA* overexpression in *K. pneumoniae* is important in reducing tigecycline and polymyxin susceptibility, but implies that our pAC*ramA* construct was not overexpressing *ramA* due to the dissimilar phenotypes observed. Furthermore, introduction of pAC*ramA* into *E. coli* DH10β failed to reduce susceptibility to tigecycline, PxB or colistin, despite George et al. [101] previously demonstrating that introduction of *ramA* from a MDR *K. pneumoniae* mutant into *E. coli* K12 produced a MDR phenotype identical to the original *K. pneumoniae* isolate. Previous work in our laboratory has also demonstrated that pAC*ramA* complementation with Ecl8Δ*ramA* returned the susceptibility phenotype to that of w/t Ecl8 (Unpublished data). The inability of our pAC*ramA* strains to therefore reduce susceptibility to tigecycline or polymyxins suggests that an error may have occurred somewhere in the process of extracting pAC*ramA* and transforming it into Ecl8 and Ecl8Δ*ramR*. The Km^R phenotype that indicated a successful transformation may have been acquired from contamination or selective pressure, leading to the assumption that Ecl8/pAC*ramA* and Ecl8Δ*ramR*/pAC*ramA* were valid. Alternatively, plasmid-mediated *ramA* overexpression may have overwhelmed transformed bacteria, potentially causing toxicity and compromising the MDR phenotype.

6.1. Summary

Both RamA and PhoPQ regulate the expression of *lpxO* in *K. pneumoniae* [1, 183, 206], whilst LpxO-mediated lipid A modifications are associated with reduced susceptibility to the last-line polymyxin drugs and host immune CAMPs [183, 206, 211]. Herein we have corroborated previous reports that overexpression of *lpxO* reduces susceptibility to PxB and colistin. Confirmation of our findings and our hypothesis will require repetition of experiments and further characterisation in the future. In fact, whilst we demonstrate a role for *ramA* in reduced PxB and colistin

susceptibility, as reported previously [1], our most convincing findings were that overexpression of *phoP* is the primary driver of reduced susceptibility to both of these agents. Due to the increasing reliance on colistin for the treatment of MDR *K. pneumoniae* infections, further investigation into the mechanisms behind *phoP*-mediated reduced susceptibility to these drugs is crucial. Furthermore, we also report that *lpxO* overexpression plays a role in reducing tigecycline susceptibility. Mutations to LPS biosynthesis genes not including *lpxO* have been observed to reduce tigecycline sensitivity in *E. coli* [239]; however we are the first to demonstrate this phenotype in *K. pneumoniae* as a result of *lpxO* overexpression. This has implications for the use of this drug in the treatment of MDR *K. pneumoniae* infections, due to the potential for intrinsically generated resistance to develop, and therefore the role of *lpxO* in this phenotype requires further characterisation.

7. References

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